TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. § 371		Attorney's Docket Number 044508-5003 US U.S. Application No. Unassigned 10/089175
International Application. No.	International Filing Date	Priority Date Claimed
PCT/US00/26504	September 27, 2000	September 27, 1999

Title of Invention: ENGINEERED RADIATION RESISTANT BIOREMEDIATING BACTERIA

Applicants For EO/EO/US: Michael J. DALY and Lawrence WACKETT

Applicants herewith submit to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. ☒ This is a FIRST submission of items concerning a filing under 35 U.S.C. § 371.
2. ☐ This is a SECOND or SUBSEQUENT submission of items concerning a filing under 35 U.S.C. § 371.
3. ☐ This express request to begin national examination procedures (35 U.S.C. § 371(f)) at any time rather than delay examination until the expiration of the applicable time limit set in 35 U.S.C. § 371(b) and PCT Articles 22 and 39(1).
4. ☒ A proper Demand for International Preliminary Examination was made by the 19th month from the earliest claimed priority date.
5. ☒ A copy of the International Application as filed (35 U.S.C. § 371(c)(2))
   a. ☐ is transmitted herewith (required only if not transmitted by the International Bureau).
   b. ☒ has been transmitted by the International Bureau.
   c. ☐ is not required, as the application was filed in the United States Receiving Office (RO/US).
6. ☐ A translation of the International Application into English (35 U.S.C. § 371(c)(2)).
7. ☒ Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. § 371(c)(3)).
   a. ☐ are transmitted herewith (required only if not transmitted by the International Bureau).
   b. ☐ have been transmitted by the International Bureau.
   c. ☐ have not been made; however, the time limit for making such amendments has NOT expired.
   d. ☒ have not been made and will not be made.
8. ☐ A translation of the amendments to the claims under PCT Article 19 (35 U.S.C. § 371(c)(3)).
9. ☐ An oath or declaration of the inventors (35 U.S.C. § 371(c)(4)).
10. ☐ A translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. § 371(c)(5)).

Items 11. to 14. below concern other document(s) or information included:

11. ☒ An Information Disclosure Statement under 37 C.F.R. § 1.97 and § 1.98.
12. ☐ An assignment document for recording. A separate cover sheet in compliance with 37 C.F.R. § 3.28 and § 3.31 is included.
13. ☒ A FIRST preliminary amendment.
    ☐ A SECOND or SUBSEQUENT preliminary amendment.
14. ☐ Other items or information: WIPO Publication WO 01/23526 (Cover page only)
    PCT/IB/304
    PCT/IB/308
    PCT/IB/332
    PCT/ISA/210

10/089175 ,080702

JC13 Rec'd PCT/PTO 27 MAR 2002

U.S. APPLICATION NO.	INTERNATIONAL APPLICATION NO.	ATTORNEY DOCKET NUMBER
10/089175 Unassigned	PCT/US00/26504	044508-5001 US

15. ☒ The following fees are submitted:

Basic National Fee (37 C.F.R. § 1.492(a)(1)-(5)):

Search Report has been prepared by the EPO or JPO.....$890.00
International preliminary examination fee paid to USPTO (37 C.F.R. § 1.482).....$710.00
No international preliminary examination fee paid to USPTO (37 C.F.R. § 1.482) but international search fee paid to USPTO (37 C.F.R. § 1.445(a)(2)).....$740.00
Neither international preliminary examination fee (37 C.F.R. § 1.482) nor international search fee (37 C.F.R. § 1.445(a)(2)) paid to USPTO.....$1,040.00
International preliminary examination fee paid to USPTO (37 C.F.R. § 1.482) and all claims satisfied provisions of PCT Article 33(2)-(4).....$100.00

ENTER APPROPRIATE BASIC FEE AMOUNT = $710.00

Surcharge of $130.00 for furnishing the oath or declaration later than ☐ 20 ☒ 30 months from the earliest claimed priority date (37 C.F.R. § 1.492(e)). $

Claims	Number Filed	Number Extra	Rate	
Total Claims	41 - 20 =	21	X $18.00	$378.00
Independent Claims	3 - 3 =	0	X $84.00	$
Multiple dependent claim(s) (if applicable)			+ $280.00	$
			TOTAL OF ABOVE CALCULATIONS	$1088.00
			Reduction by ½ for filing by small entity	-$544.00
			SUBTOTAL =	**$544.00**
Processing fee of $130.00 for furnishing the English translation later than ☐ 20 ☐ 30 months from the earliest claimed priority date (37 C.F.R. § 1.492(f)).				+$
			TOTAL NATIONAL FEE =	**$544.00**
Fee for recording the enclosed assignment (37 C.F.R. § 1.21(h)). The Assignment must be accompanied by an appropriate cover sheet (37 C.F.R. §§ 3.28, 3.31). $40.00 per property				$
			TOTAL FEES ENCLOSED =	**$544.00**
			Amount to be refunded	$
			Amount to be charged	$544.00

a. ☒ Please charge Deposit Account No. 50-0310 in the amount of **$544.00** to cover the above fees. A duplicate copy of this sheet is enclosed.

b. ☒ **Except** for issue fees payable under 37 C.F.R. § 1.18, the Commissioner is hereby authorized by this paper to charge any additional fees during the entire pendency of this application including fees due under 37 C.F.R. § 1.16 and § 1.17 which may be required, or credit any overpayment to Deposit Account No. 50-0310.

Customer No. 09629
SEND ALL CORRESPONDENCE TO:
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1111 Pennsylvania Avenue, N.W.
Washington, D.C. 20004
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Robert Smyth
Reg. No. 50,801

Submitted: March 27, 2002

PATENT
Attorney Docket **044508-5003**

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application: **Michael J. Daly *et al.***
U.S. Application No. **Not Assigned** — Group Art Unit: **Not Assigned**
Date of National Stage Entry: **March 27, 2002** — Examiner: **Not Assigned**
Based on **PCT/US00/26504**
Filed: **September 27, 2000**
For: **Engineered Radiation Resistant Bioremediating Bacteria**

PRELIMINARY AMENDMENT

Prior to the examination of the above-identified application, please amend the application as follows:

In the Claims:

Please replace claims 25, 32, 38 and 41 with the following substitute claims.

25. A bioremediation composition comprising the bacterium of claim 1.

32. A method of bioremediation, comprising the step of exposing a sample to the composition of claim 26.

38. A method of bioremediation, comprising the step of exposing a sample to the composition of claim 35.

41. A radiation resistant bacterium of claim 1, wherein the bacterium is selected from the group consisting of *D. radiodurans*, *D. radiopugnans*, *D. grandis*, *D. proteolyticus*, *D. murrayi*, *D. geothermalis*, and *D. radiophilus*.

Remarks

Applicants respectfully submit that no new prohibited matter has been introduced by this Preliminary Amendment. The amendments to the claims removed multiple dependencies among the claims.

Attached hereto is a marked-up version of the changes made to the specification by the current amendment. The attached page is captioned "Version with markings to show changes made" as required by revised rules for claim amendments.

If there are any other fees due in connection with the filing of this Preliminary Amendment, please charge the fees to our Deposit Account No. 13-4520. If a fee is required for an extension of time under 37 C.F.R. 1.136 not accounted for above, such an extension is requested and the fee should also be charged to our Deposit Account.

Respectfully submitted,
Morgan, Lewis & Bockius LLP

Robert Smyth
Registration No. 50, 801

Date: **March 27, 2002**
Morgan, Lewis & Bockius LLP
Customer No. **009629**
1111 Pennsylvania Avenue, N.W.
Washington, D.C. 20004

VERSION WITH MARKINGS TO SHOW CHANGES MADE

In the claims:

Claim 25 has been amended as follows:

25. A bioremediation composition comprising the [~~a~~] bacterium of claim [~~any one of claims~~] 1 [~~-24~~].

Claim 32 has been amended as follows:

32. A method of bioremediation, comprising the step of exposing a sample to the [~~a~~] composition of claim [~~any one of claims~~] 26 [~~-28~~].

Claim 38 has been amended as follows:

38. A method of bioremediation, comprising the step of exposing a sample to the [~~a~~] composition of claim [~~any one of claims~~] 35 [~~-37~~].

Claim 41 has been amended as follows:

41. A radiation resistant bacterium of claim [~~any one of claims~~] 1 [~~-4 and 16-24~~], wherein the bacterium is selected from the group consisting of *D. radiodurans*, *D. radiopugnans*, *D. grandis* , *D. proteolyticus*, *D. murrayi*, *D. geothermalis*, and *D. radiophilus*.

FIG. 1

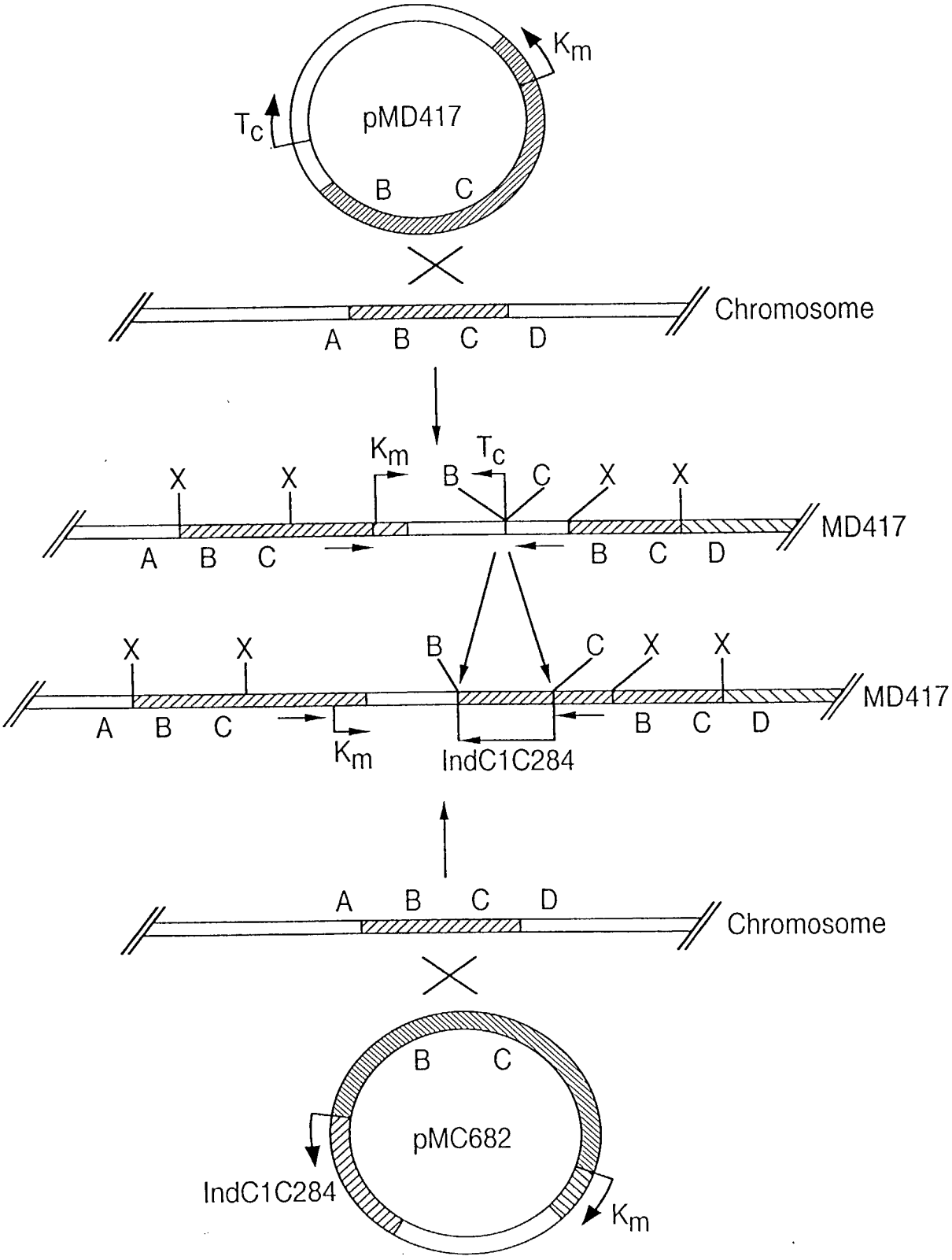
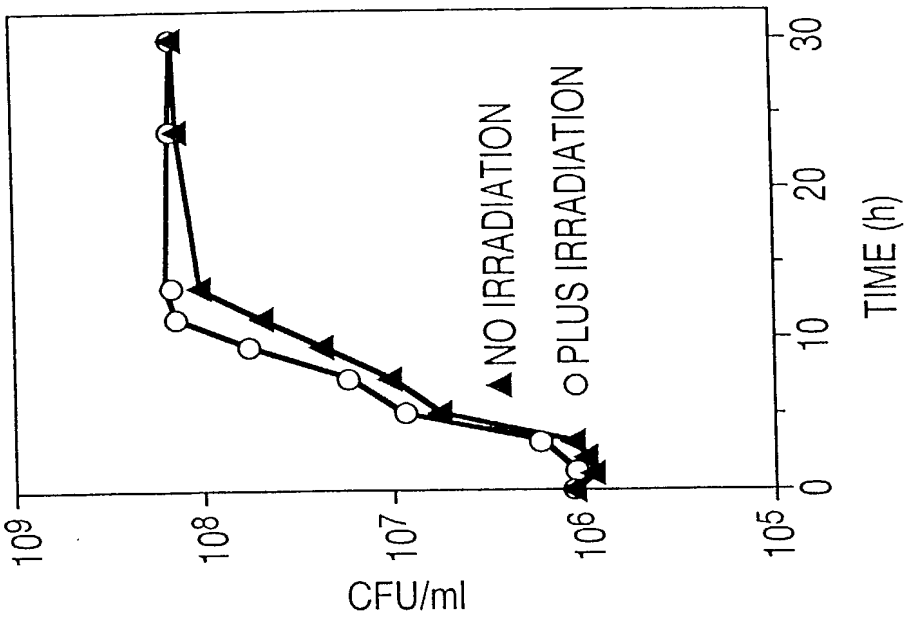

Km
Tc
pMD417
B
C
A
B
C
D
Chromosome
Km
Tc
X
X
B
C
X
X
A
B
C
B
C
D
MD417
X
X
B
C
X
X
A
B
C
Km
IndC1C284
B
C
D
MD417
A
B
C
D
Chromosome
B
C
pMC682
IndC1C284
Km

FIG. 2B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PLUS IRRADIATION
CFU/ml
TIME (h)
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10^8
10^7
10^6
10^5
0
10
20
30

FIG. 2A

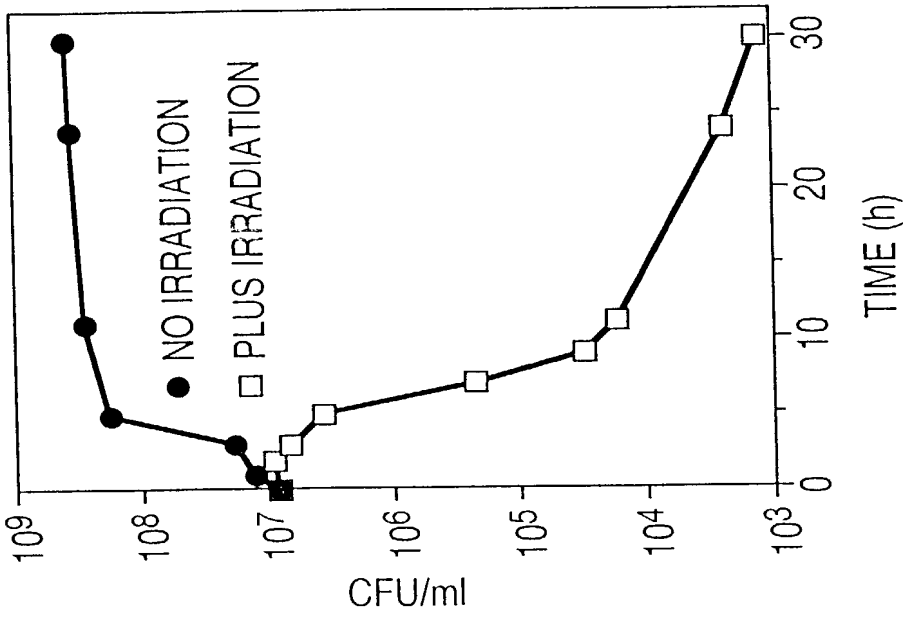

NO IRRADIATION
PLUS IRRADIATION
CFU/ml
TIME (h)
10^9
10^8
10^7
10^6
10^5
10^4
10^3
0
10
20
30

FIG. 3

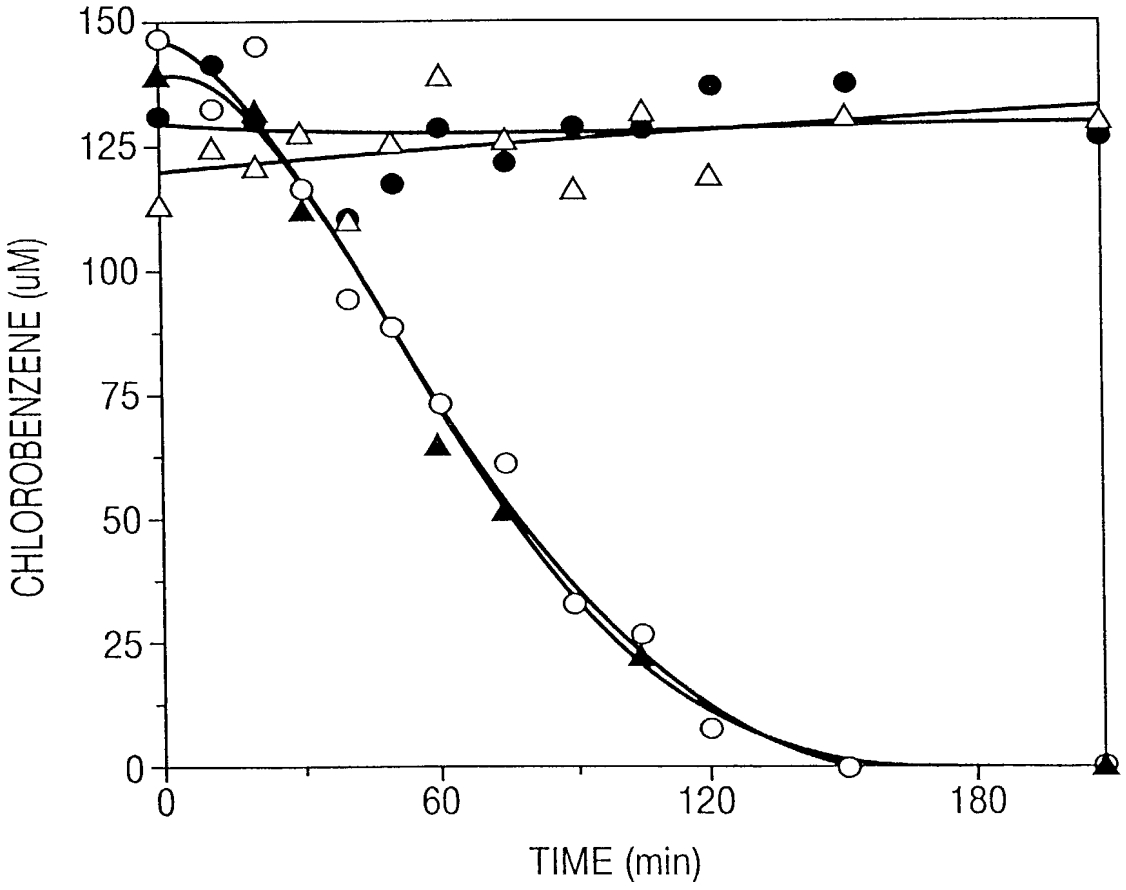


FIG. 4

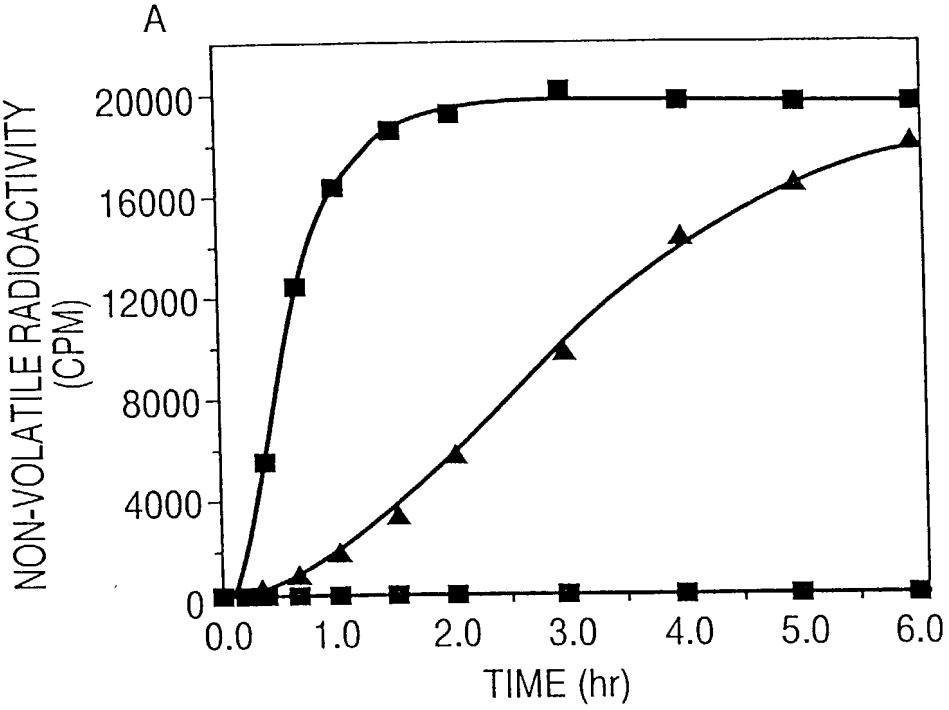


FIG. 5

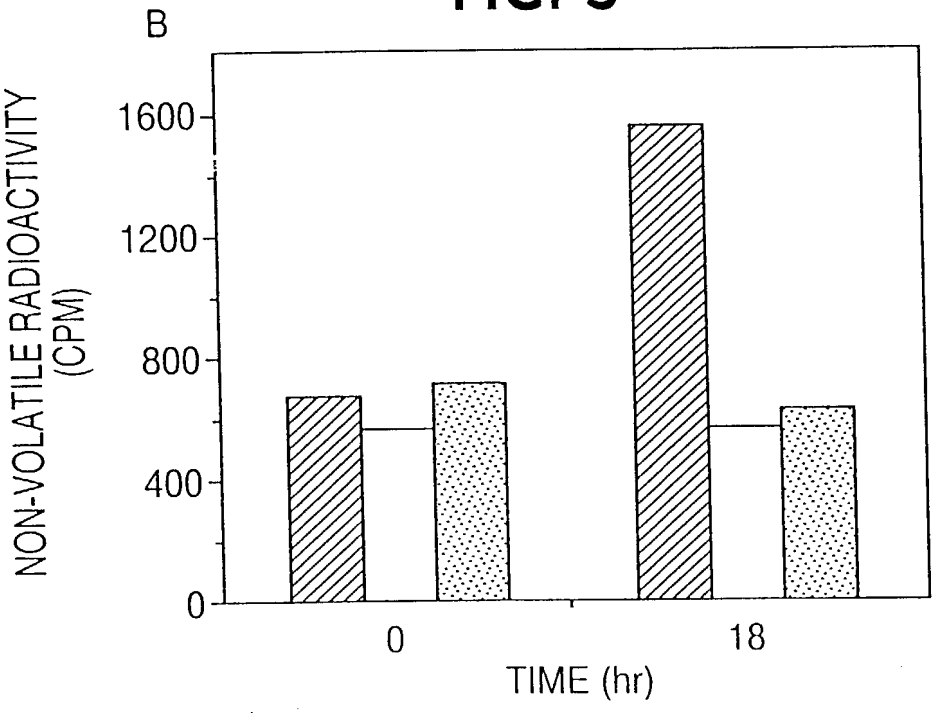
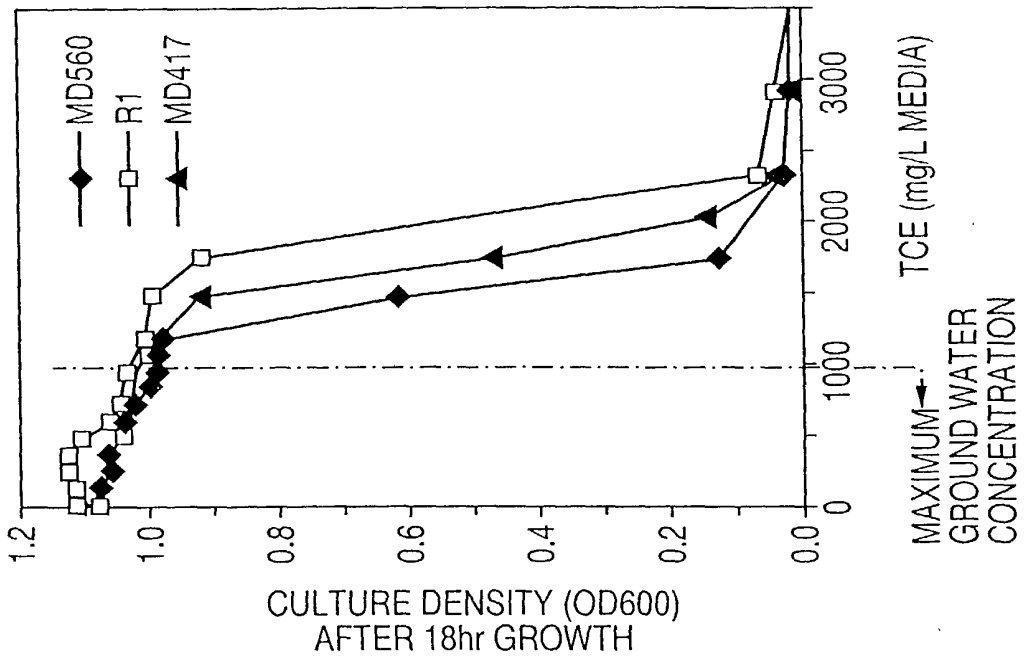


FIG. 6B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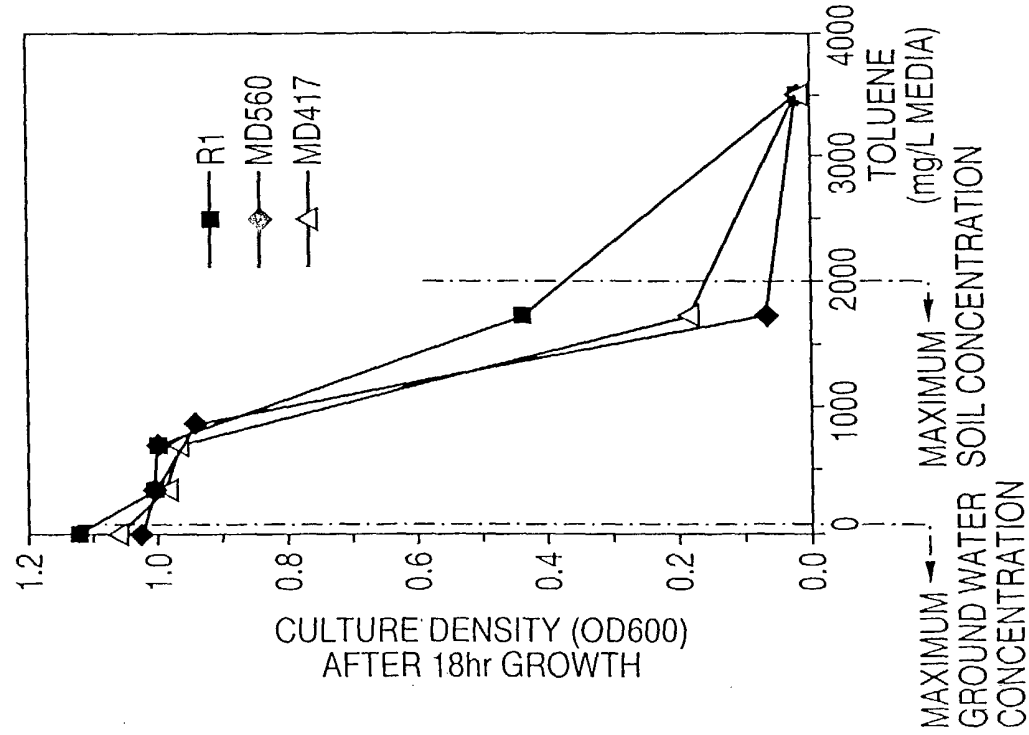
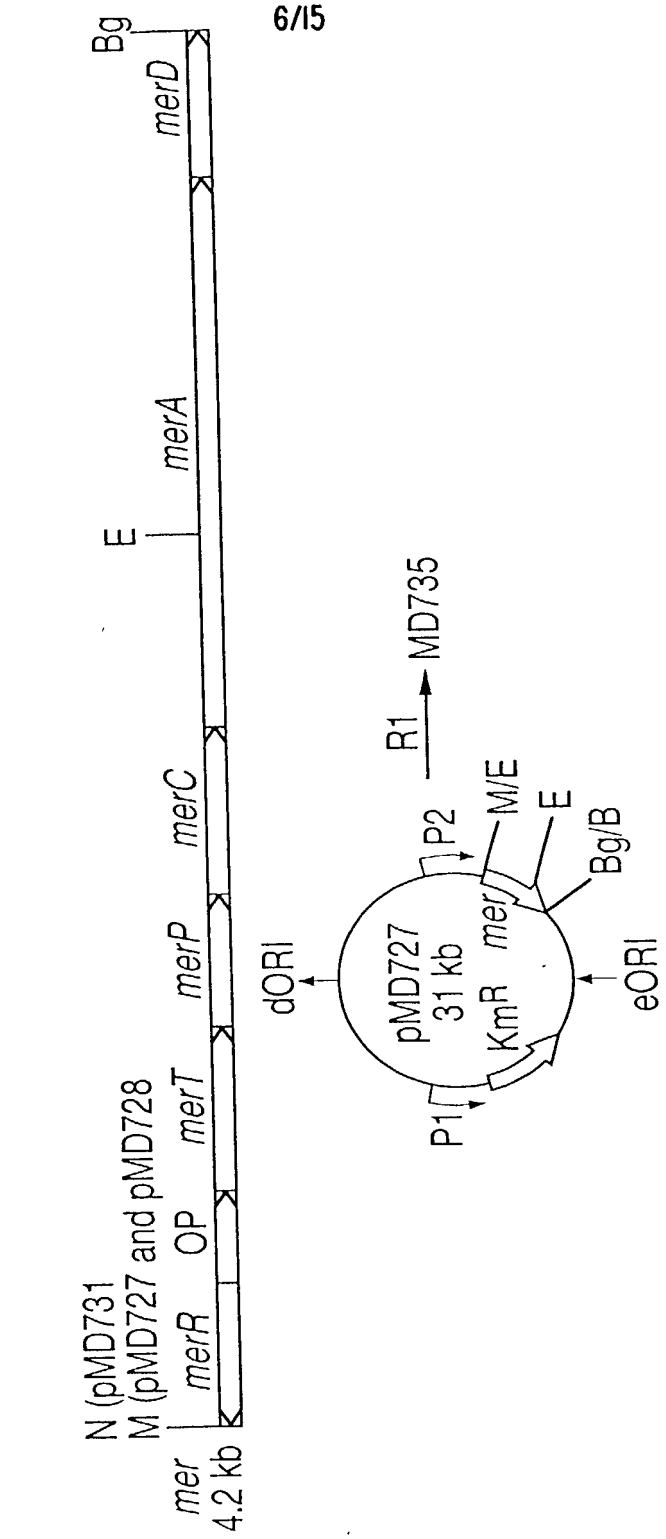
R1
MD417
1.2
1.0
0.8
0.6
0.4
0.2
0.0
0
1000
2000
3000
TCE (mg/L MEDIA)
MAXIMUM GROUND WATER CONCENTRATION
CULTURE DENSITY (OD600) AFTER 18hr GROWTH

FIG. 6A


R1
MD560
MD417
1.2
1.0
0.8
0.6
0.4
0.2
0.0
0
1000
2000
3000
4000
TOLUENE (mg/L MEDIA)
MAXIMUM SOIL CONCENTRATION
MAXIMUM GROUND WATER CONCENTRATION
CULTURE DENSITY (OD600) AFTER 18hr GROWTH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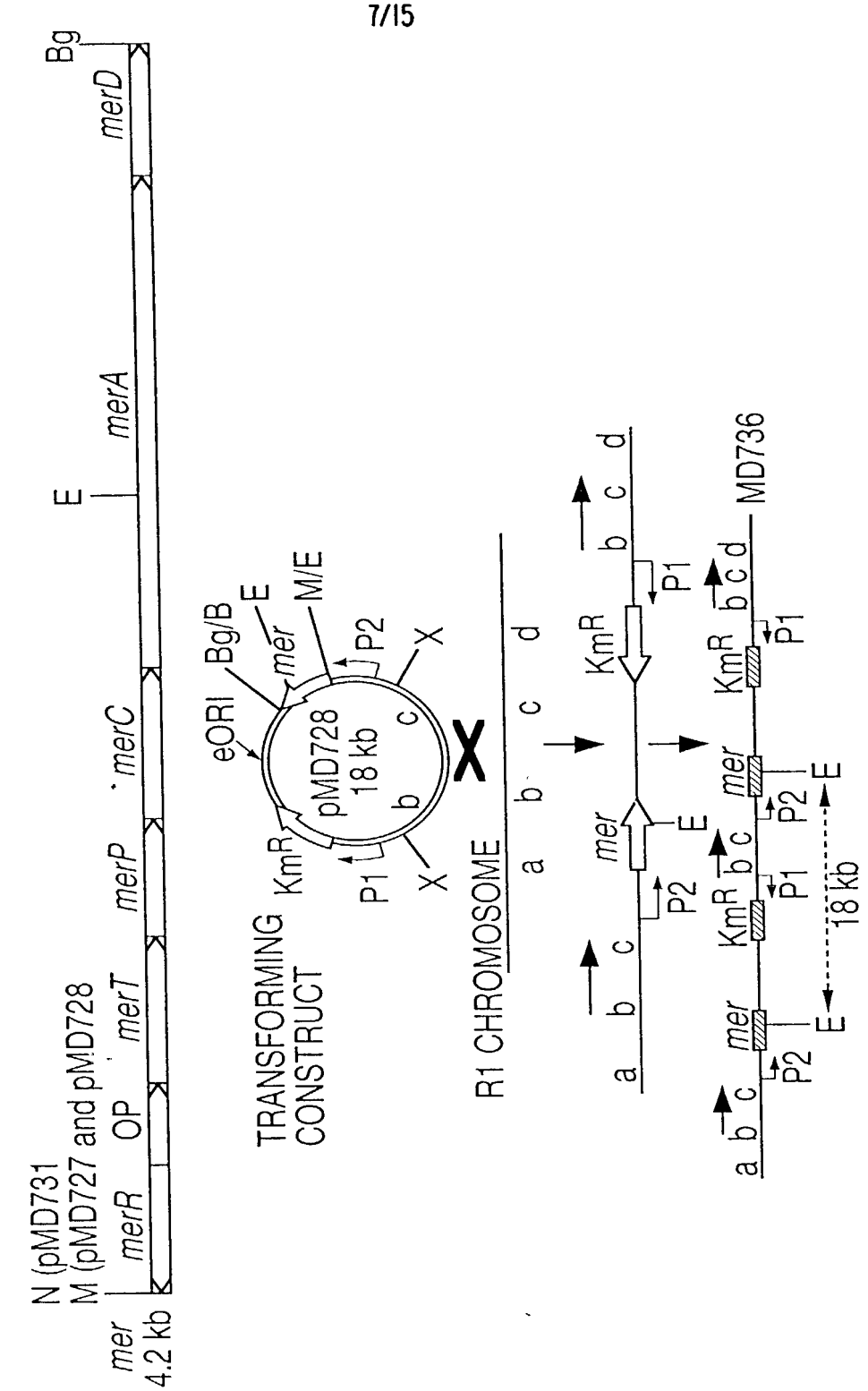
4.2 kb
N (pMD731
M (pMD727 and pMD728
merR
OP
merT
merP
merC
E
merA
Bg
merD
dORI
pMD727
31 kb
KmR
merI
P1
P2
R1
MD735
M/E
E
Bg/B
eORI

FIG. 7A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M (pMD727 and pMD728
mer
4.2 kb
merR
OP
merT
merP
merC
merA
merD
Bg
E
TRANSFORMING
CONSTRUCT
KmR
eORI
Bg/B
E
mer
M/E
P2
X
pMD728
18 kb
P1
R1 CHROMOSOME
MD736
18 kb

FIG. 7B

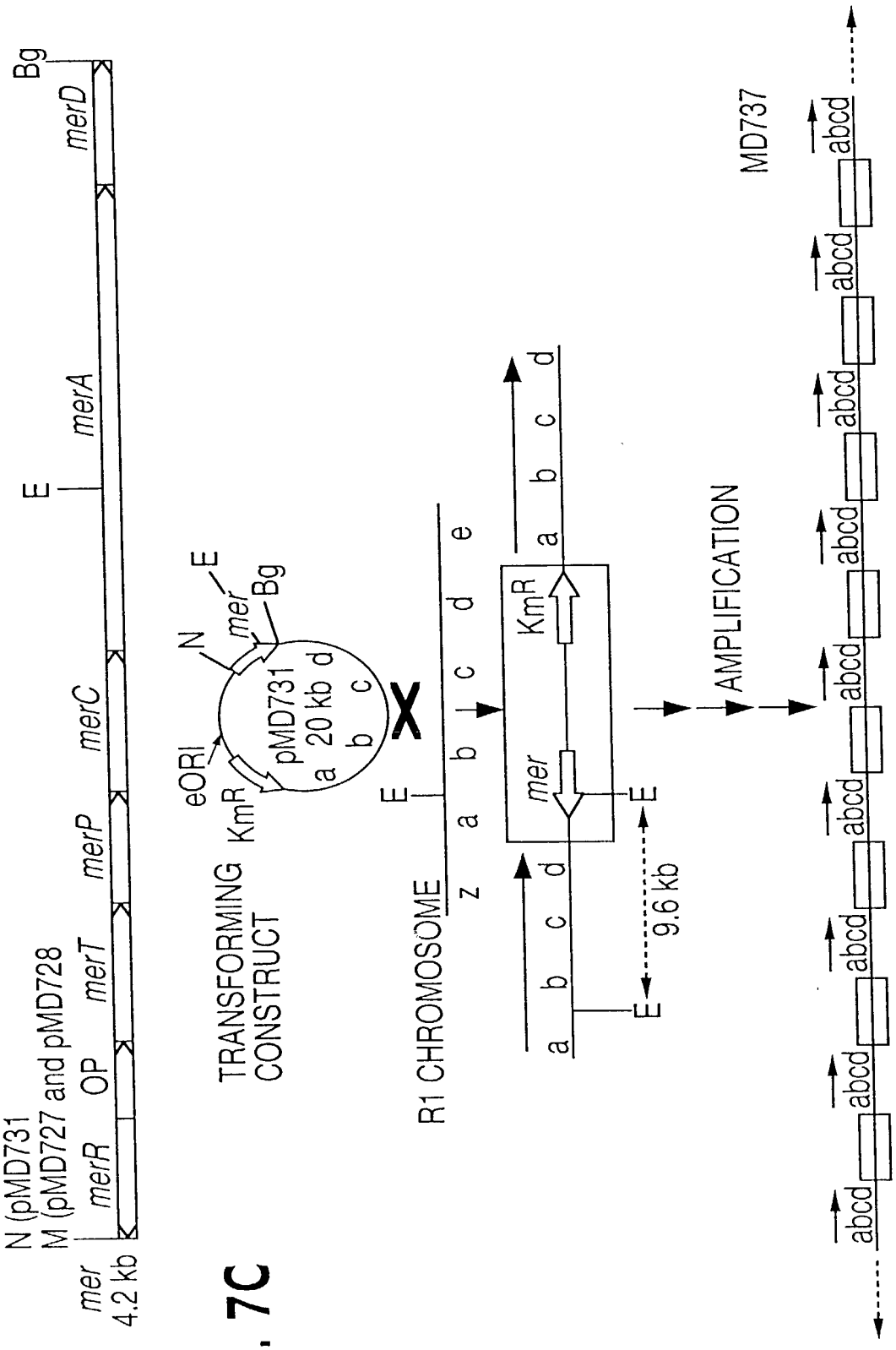

N (pMD731
M (pMD727 and pMD728
mer
4.2 kb
merR
OP
merT
merP
merC
merA
E
merD
Bg
TRANSFORMING
CONSTRUCT
eORI
KmR
pMD731
20 kb
a
b
c
d
N
mer
E
Bg
X
R1 CHROMOSOME
z
a
b
c
d
e
E
KmR
mer
9.6 kb
AMPLIFICATION
MD737
abcd

FIG. 7C

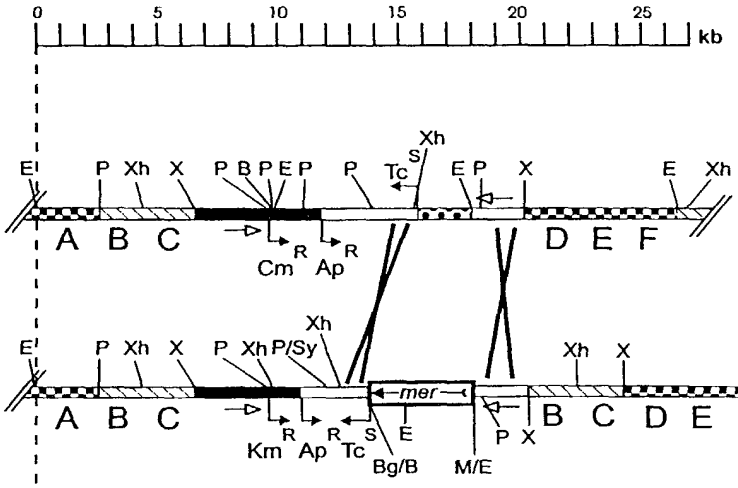


FIG. 8A

FIG. 8B

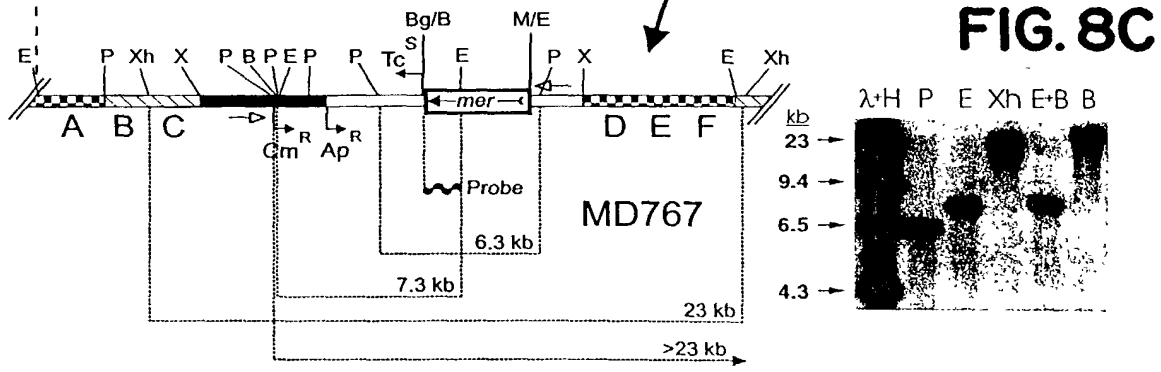


FIG. 8C

FIG. 9A

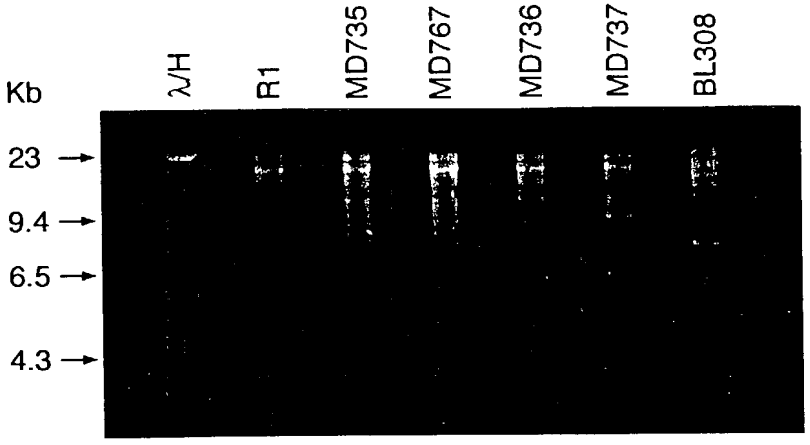


FIG. 9B

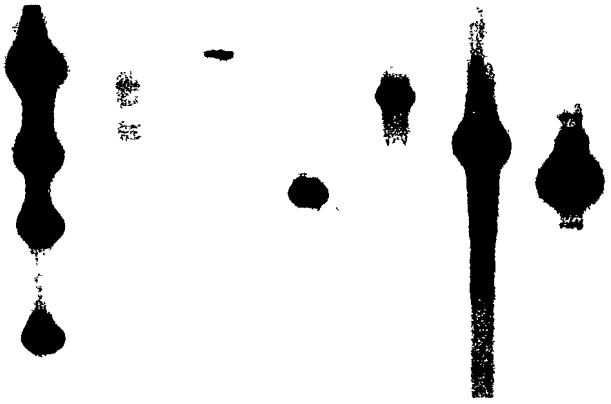


FIG. 9C

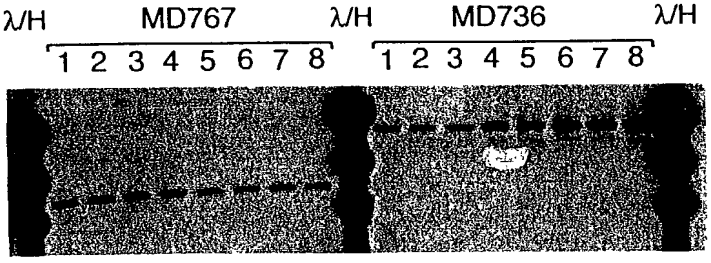


FIG. 9D

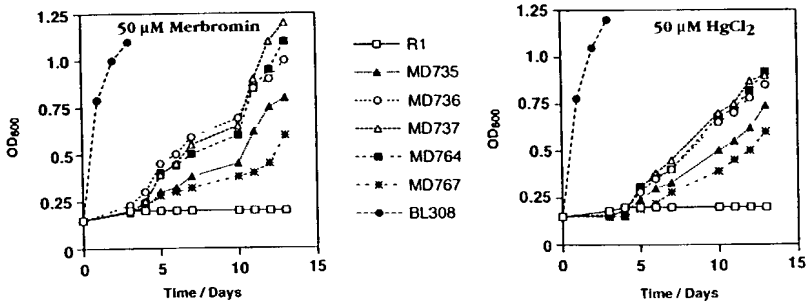


FIG. 10

FIG. IIA

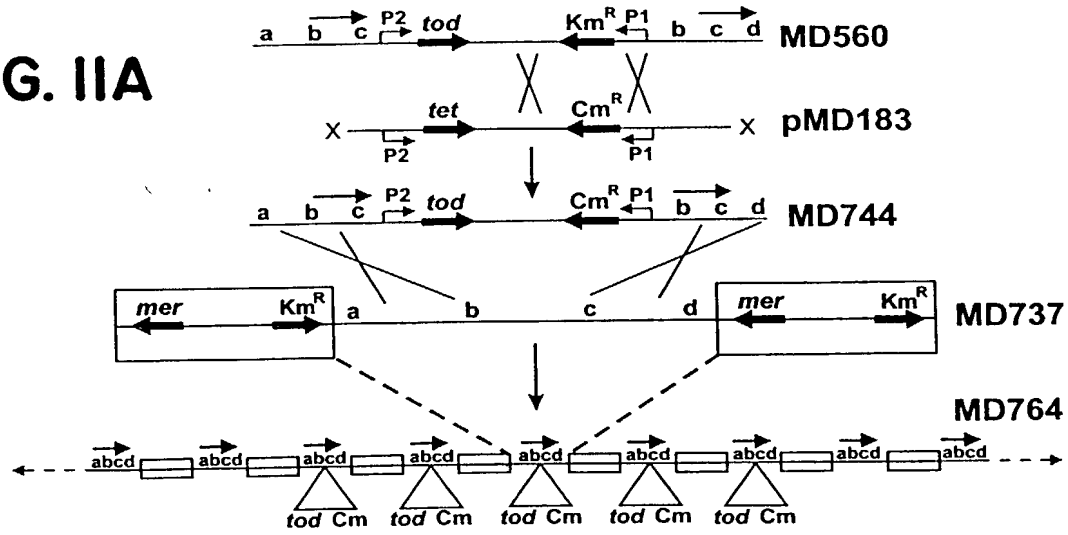


FIG. IIB

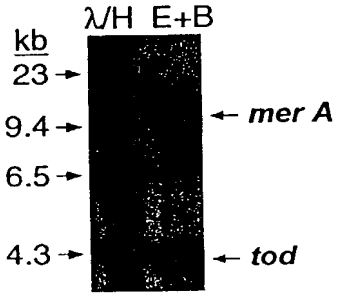


FIG. IIC

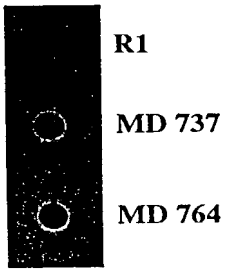


FIG. IID

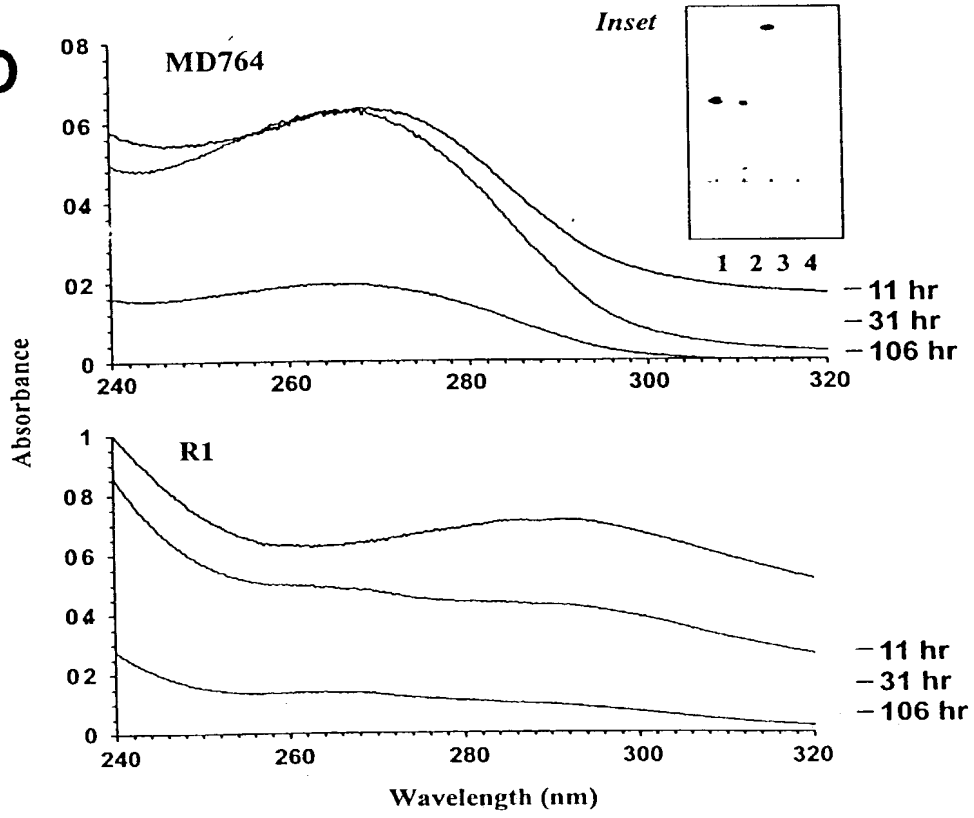


FIG. 12A

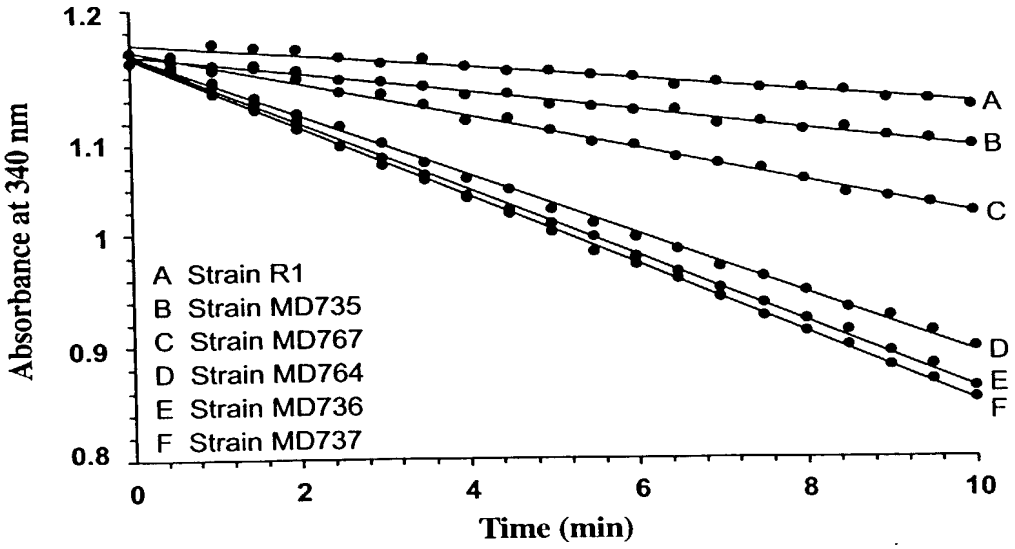


FIG. 12B

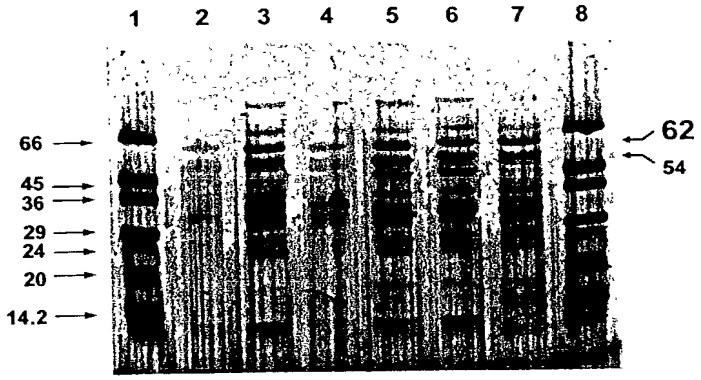


FIG. 12C

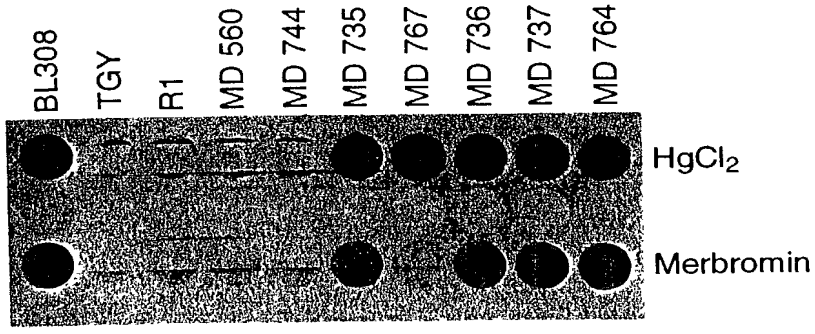


FIG. 13

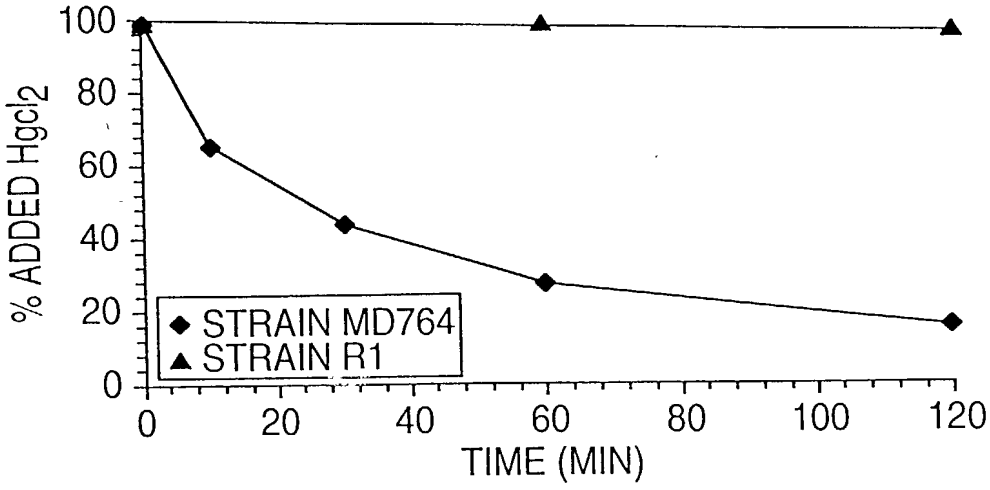
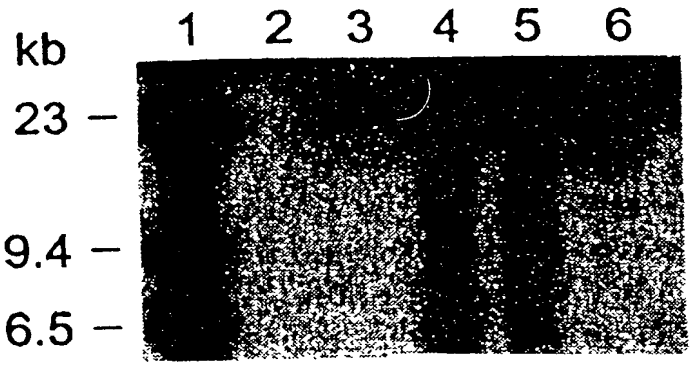

100
80
60
40
20
0
% ADDED Hgcl2
STRAIN MD764
STRAIN R1
0
20
40
60
80
100
120
TIME (MIN)

FIG. 14



15/pr/p

ENGINEERED RADIATION RESISTANT BIOREMEDIATING BACTERIA

INVENTORS: Michael J. Daly and Lawrence P. Wackett

FEDERAL SUPPORT

This work was funded, in part, by grants DE-FG07-97ER20293 and DE-FG02-97ER62492 from the U.S. Department of Energy, the applications for which are herein incorporated by reference in their entirety.

FIELD OF THE INVENTION

The invention relates generally to the production of radiation resistant microorganisms which are useful bioremediation agents. Preferred microorganisms include *Deinococcus* species, including *D. radiodurans* and *D. geothermalis* strains that have been engineered to metabolize, degrade or detoxify inorganic and organic contaminants such as radionuclides, heavy metals and organic solvents. This application claims priority to U.S. Provisional Application 60/155,767, filed September 27, 1999 which is herein incorporated by reference in its entirety.

BACKGROUND OF THE INVENTION

Many of the solid and liquid wastes generated as a result of global nuclear weapons production between 1945 and 1986 were discharged to the ground and are now contaminating the subsurface at many sites. These wastes contain inorganic and organic contaminants that include radionuclides, heavy metals, acids/bases, and solvents (Riley *et al.*, 1992). In the United States alone, it is estimated that these leaking buried wastes (3×10^6 m^3) have contaminated 7.5×10^7 m^3 of surface and subsurface soils and about 2×10^{12} dm^3 of groundwater (Office of Energy Research, DOE, 1992). With the end of the Cold War in the early 1990's, the United States Department of Energy (DOE) shifted its emphasis from nuclear weapons production to cleanup of its radioactive waste. This remediation effort is now the largest program of its kind ever undertaken by the United States (Macilwain, 1996).

In 1992, the DOE surveyed a representative 91 out of 3,000 contaminated sites at eighteen U.S. research facilities (Riley *et al.*, 1992). Site-characterization activities at those 91 sites have recorded large inventories of chemical and radioactive contaminants in the soils, sediments and ground waters surrounding these disposal sites (Riley *et al.*, 1992). The most common metallic contaminants from DOE wastes that have been found in ground waters include the radionuclides ^{235}Uranium $(\gamma, \alpha)^E$, ^{90}Strontium $(\beta^-)^E$, ^{238}Plutonium $(\alpha)^E$, ^{137}Cesium $(\gamma, \beta^-)^E$, and ^{99}Technetium $(\beta^-)^E$; and the metals, Chromium, Lead and Mercury (Riley *et al.*, 1992, McCullough *et al.*, 1999). A more limited soil/sediment characterization has shown these same constituents throughout soil profiles and sediments (Riley *et al.*, 1992). One third of the ninety-one sites are radioactive with some reported radiation levels as high as 10 mCi/L, within or close to the contaminating source. These high radiation levels are extremely damaging to living organisms over extended periods, often resulting in cell death.

Of the 3,000 waste sites disclosed by DOE, the total cleanup cost, by physicochemical methods, was estimated in 1988 to be about $90 billion (U.S. Government Accounting Office, GAO, 1988) and more recently between $189 and $265 billion, over a seventy year period (1996 Baseline Environmental Management Report (visited September 27, 1999) <http://www.em.doe.gov/bemr96/>). DOE budget projections for cleanup activities for just the next ten years exceed $60 billion (MuCullough *et al.*, 1999). These sites, therefore, represent defined targets for less expensive *in situ* bioremediation technologies utilizing specialized microorganisms that can remediate both metallic and organic contaminants. The utility of microbiological methods for the treatment of highly radioactive waste environments will largely be determined by the ability of microorganisms catalyzing the desired function(s), to survive and function under radiation stress.

Numerous microorganisms (including *Shewanella*, *Geobacter* and *Pseudomonas* spp.) have been described, and studied in detail, for their ability to transform, detoxify, or immobilize, a variety of organic and metallic pollutants (Gorby *et al.*, 1992; Higham *et al.*, 1984; Ji *et al.*, 1992; Lovely, 1995; Nies *et al.*, 1995; Tsapin *et al.*, 1996; Turner *et al.*, 1995; Voordouv *et al.*, 1996). Detoxification of the toxic compounds and metals at these

sites is an important goal in remediating or stabilizing contaminated sites as well as preventing further dissemination. Generally, microorganisms are sensitive to the damaging effects of ionizing radiation, and most of the bacteria currently being studied as candidates for bioremediation are no exception. For example, *Pseudomonas* spp. is very sensitive to radiation (more sensitive than *E. coli* [Thornley, 1963]) and is not suited to remediate radioactive wastes. Therefore, radiation resistant microorganisms that can remediate toxic metals need to be identified in nature or engineered in the laboratory to address this problem.

SUMMARY OF THE INVENTION

The present invention is based in part on the discovery that the most radiation resistant organism yet discovered, *Deinococcus*, can be engineered to express heterologous enzymes capable of detoxifying or metabolizing organic compounds, heavy metals and radionuclides.

The invention includes radiation resistant bacteria engineered to detoxify at least one toxin, preferably radiation resistant strains which survive acute exposure to ionizing radiation of up to about 15,000 Gy or can grow in the presence of continuous radiation of about 60 Gy/hour, most preferably, radiation resistant strains of *Deinococcus* engineered to detoxify at least one toxin, such as radionuclides, heavy metals and organic compounds.

The invention also includes radiation resistant bacterial strains engineered to detoxify at least two toxins. Radiation resistant bacteria of the invention include *Deinococcus* strains engineered to express a heterologous protein or enzyme selected from the group consisting of toluene dioxygenase, the proteins encoded by the *mer* operon, the proteins encodes by the *Pseudomonas tol* region, the proteins encoded by the *xyl*L-*xyl*E operon, a monooxygenase, the proteins encoded by *bph*A1A2A3A4, the proteins encoded by *czc*A, B and C genes, the proteins encoded by a *cyt*c3, the protein encoded by the *smt*A *abd*B genes and the *ars*A and B genes

The invention also includes bioremediation compositions comprising at least one radiation resistant bacterial strain of the invention. Such bioremediation compositions may contain, in addition to the bacterial strains of the invention and other compounds or

diluents, agents selected from the group consisting of film forming agents and nutrient agents. Bioremediation compositions of the invention may also be formulated for controlled release.

A further embodiment of the invention includes methods of bioremediation, comprising the step of exposing a sample to a bioremediating composition of the invention. The compositions of the invention may also be released into an industrial or other waste site.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1. Regional chromosomal maps and functions of *D. radiodurans* strains MD417 (*tod*$^-$) and MD560 (*tod*$^+$). Construction of these strains is described in the Experimental Protocol. MD560 constitutively expresses TDO (encoded by *todC1C2BA*). MD417 is a control strain (lacking *tod* genes). The strains are the products of transformation of wild-type strain R1 with the circular plasmids pMD532 and pMD417, respectively. The two arrows drawn between chromosomal regions MD417 and MD560 show the location of the *tod* genes; the black arrow below the *tod* genes shows the direction of functional transcription. The checkered segment **BC** indicates the chromosomal integration sequence. **A** and **D** are chromosomal sequences flanking the integration site **BC**. Km (resistance to kanamycin) is encoded by the *aphA* gene [diagonally hatched segment]. Transcription of the *aphA* genes is driven by a *Deinococcal* constitutive promoting sequence (open arrow) located in the black segments. Tc (resistance to tetracycline) is encoded by the *tet* gene [white region]. Transcription of the *tet* and *todC1C2BA* genes is driven by another *Deinococcal* constitutive promoting sequence (open arrow) present in the light grey segments. Restriction sites: X, *Xba*I; B, *Bam*HI; E, *Eco*RI.

Figure 2. Effect of γ-irradiation on the growth of *E. coli* (left) and *D. radiodurans* R1 (right). *E. coli* (wildtype) and *D. radiodurans* R1 (wildtype) were both grown to the

plateau phase of their respective growth cycles and diluted 150-fold in fresh growth media. The diluted cultures were divided into two parts and incubated with aeration in the presence and absence of γ–radiation (^{137}Cs; 60 Gy/hr) for a total of thirty hours. The survival rates were determined by plating appropriate dilutions of irradiated cells and counting the number of colony forming units (cfu) following incubation.

Figure 3. Effect of γ-irradiation on the synthesis and function of TDO expressed in *D. radiodurans*. Strain MD560 (*tod*$^+$) in the presence of (solid triangle) and absence of (open circle) irradiation; and MD417 (*tod*$^-$) in the presence of (solid circle) and absence of (open triangle) irradiation. Initially, strains MD560 and MD417 were grown in the presence and absence of γ-irradiation (60 Gy/hr) for sixteen hours to the plateau phase, in the absence of chlorobenzene. Cultures were then diluted with fresh medium and exponentially growing cells were harvested following continued growth in the presence and absence of γ-radiation (60 Gy/hr). Cells were then concentrated and incubated with 125 μM chlorobenzene in the presence and absence of irradiation (60 Gy/hr) for the indicated time periods.

Figure 4. Incubation of ^{14}C-labeled toluene with *D. radiodurans* strains MD560 (*tod*$^+$; closed triangle) and MD417 (*tod*$^-$; open square), *E. coli* (pDTG351; *tod*$^+$; solid square), and negative control of TGY medium alone (open circle). Detection of ^{14}C non-volatile product is as described in Experimental Protocol.

Figure 5. Detection of non-volatile ^{14}C-labeled material in media containing cells after an 18-hour incubation with ^{14}C-trichloroethylene. *D. radiodurans* strain MD560 (*tod*$^+$; black), MD417 (*tod*$^-$, open), and TGY medium control (grey).

Figure 6. Effect of toluene and TCE on the growth of *D. radiodurans* strains R1, MD417 (vector control, *tod*$^-$) and MD560 (*tod*$^+$). Strains were first grown overnight in liquid growth medium (to 1.1 OD_{600}) followed by dilution into fresh growth medium (to 0.02 OD_{600}) containing varying amounts of toluene (left) and TCE (right). After eighteen hours

of incubation at 32°C, the cell densities of the cultures were determined and plotted as a function of solvent concentration.

Figure 7. Plasmid and chromosomal maps. Top, 4.2 kb *mer* operon of pBD7 (Barrineau *et al.*, 1984) encoding six proteins: MerR, activation/repression of the *mer* operon; MerT, mercuric ion transport protein; MerP, periplasmic mercuric ion binding protein; MerC, transmembrane protein; MerA, mercuric reductase; and MerD, putative secondary regulatory protein. OP, operator/ promoter sequence. **A)** The *Stu*I site at the end of the *mer* operon was converted to a *Bgl*II (Bg) site, yielding pMD725, followed by the conversion of the *Nco*I (N) site at the start of the operon to an *Mfe*I (M) site, yielding pMD726. The *Mfe*I-*Bgl*II (4.2 kb) fragment of pMD726, was cloned into the *Eco*RI (E)-*Bam*HI (B) site of the *D. radiodurans* plasmid pMD66 (Daly *et al.*, 1994a), yielding pMD727. pMD727 was transformed into *D. radiodurans* strain R1 (wild-type) by selection with kanamycin (Km), giving strain MD735. The specifics of DNA cloning, Southern blotting and transformations were as described in the Experimental section. dORI, *Deinococcal* origin of replication (Daly *et al.*, 1994b; 1995; 1996; 1997). eORI, *E. coli* origin of replication (Daly *et al.*, 1994a). P1 and P2 are two different constitutive *Deinococcus* promoters (Lange *et al.*, 1998). Km^R, kanamycin resistance gene *aphA*. **B)** The *Mfe*I-*Bgl*II (4.2 kb) fragment of pMD726 was cloned into the *Eco*RI-*Bam*HI site of the *D. radiodurans* tandem duplication vector pMD417 (Daly *et al.*, 1996), yielding pMD728. pMD728 was transformed into *D. radiodurans* strain R1 with Km selection, giving strain MD736. Two rounds of recombinative duplication are illustrated, yielding two vector copies on a chromosome. bc, duplicated chromosomal target sequence. X, *Xba*1. Remaining abbreviations and symbols are as in A. **C)** The unique *Dra*I site of the *D. radiodurans* amplification vector pS11 (Smith *et al.*, 1988) was converted to an *Nco*I site, yielding pMD729. The *Nco*I-*Bgl*II fragment of pMD725 was cloned into the *Nco*I-*Bgl*II site of pMD729, yielding pMD731. pMD731 was transformed into *D. radiodurans* strain R1 with Km selection, giving strain MD737. Multiple rounds of recombinative duplication are illustrated, yielding many insertions per chromosome. abcd, duplicated chromosomal target sequence. Remaining abbreviations and symbols are as in A.

Figure 8. Construction and structure of a chromosomal direct insertion of the *mer* operon. **A)** Strain MD399 (Daly *et al.*, 1995) is a previously constructed *D. radiodurans* strain containing a direct insertion of a plasmid having regions of identity with the duplication insertion in strain MD736. ABC and DEF are contiguous chromosomal sequences in wildtype *D. radiodurans* strain R1, lacking homology. BC is the duplicated chromosomal flanking region in MD736. Open-headed arrows are constitutive deinococcal promoters (Lange *et al.*, 1998). Black crosses between the MD399 and MD736 chromosomes link regions of homology and show where crossovers occurred. Cm^R, chloramphenicol resistance gene, *cat*. Km^R, kanamycin resistance gene, *aphA*. Tc^S, mutated tetracyclin gene (Daly *et al.*, 1994b), *tet*. E, *Eco*RI; P, *Pvu*II; X, *Xba*I; Xh, *Xho*I; B, *Bam*HI; P/Sy, *Pvu*II/*Sty*I fusion. mer, 4.2 kb *mer* operon. **B)** The standard transformation protocol (Daly *et al.*, 1994a) was used to introduce MD736 DNA into MD399. However, following the addition of transforming MD736 DNA and overnight incubation with MD399, 0.1 ml of the transformed cell suspension (~1 x 10^7 cells) were transferred to 0.9 ml fresh TGY liquid medium containing 15 μg/ml Merbromin. After an eighteen hour incubation with shaking at 32°C, aliquots of 100 μl of transformed cells were spread on petri plates of non-selective TGY solid medium (30 cm^3/plate). Once dry, 8 μl of 0.1 M Merbromin were pipetted onto the center of the plate. Mercury-resistant colonies grew, and were isolated from, within a zone of wildtype growth inhibition. MD399 (left, control); MD399 + MD736 DNA (right). **C)** Right, MD767 was selected and subjected to a detailed mapping of the *mer* operon integration site using restriction enzymes, Southern blotting, and probing with various radiolabeled DNA fragments, including a probe made from the *Eco*RI-*Bgl*II fragment of the *mer* operon (black wavy line). Left, The chromosomal structure of the direct chromosomal insertion containing the *mer* operon in MD767. Abbreviations and symbols are as described in A.

Figure 9. Determination of *mer* operon copy number and associated mercury resistance phenotype. **A)** Genomic DNA from each of the exponentially growing strains R1, MD735, MD767, MD736, MD737 was prepared as described in the Experimental section and previously described (Daly *et al.*, 1994a). For BL308 (*E. coli* strain K12/pDB7) genomic

DNA was prepared from stationary-phase cells. ~600 ng of each DNA sample was cut with *Eco*RI and electrophoresed at 60 volts for sixteen hours in a 0.6 % agarose gel. λ/H, lambda phage DNA cut with *Hind*III. **B)** The gel was blotted and hybridized to a radiolabeled 1.5 kb *Eco*RI-*Bgl*II fragment of pMD726, containing part of *merA* and all of *merD*. The order of lanes is as in A. The number of disintegration counts in each of the hybridizing bands was determined using the Instant Imager (Packard Instrument Company) and adjusted for DNA content present in each of the corresponding gel lanes (A), similarly scanned. **C)** Genomic DNA was prepared from strains MD767 (direct insertion) and MD736 (tandem duplication) growing in increasing concentrations of Merbromin (0-35 μM in 5 μM steps, lanes 1-8, respectively). DNA was analyzed as described in A and B. The 23 kb, 9.4 kb and 6.5 kb λ/H size markers are shown. **D)** Growth curves for each of the strains described in A and B were determined by inoculating ~5 x 10^6 cells of each into growth medium containing 50 μM Merbromin (left) or 50 μM $HgCl_2$ (right).

Figure 10. Effect of continuous exposure to γ-radiation and mercury (II) on the growth of strains containing different copy numbers of the *mer* operon. 1 x 10^5 cells of each of the *D. radiodurans* strains R1 (*recA*$^+$, *mer*$^-$), rec30 (*recA*$^-$, *mer*$^-$; Daly1), MD735 (*recA*$^+$, 1 x *mer*$^+$/cell), MD767 (*recA*$^+$, 10 x *mer*$^+$/cell), MD736 (*recA*$^+$, 10-20 *mer*$^+$/ cell), MD737 (*recA*$^+$, 150 *mer*$^+$/cell), and the wildtype *E. coli* strain K12 (*recA*$^+$) containing pDB7 (BL308; [24]) (20-30 x *mer*$^+$/cell) were spotted onto two TGY agar plates (**A** and **B**) and two TGY agar pates containing 30 μg/ml Merbromin (**C** and **D**). Following plate inoculation, one of these plain TGY plates (**B**) and one of the TGY plus Merbromin plates (**D**) were placed into the ^{137}Cs irradiator (60 Gy/hour) (Gammacell 40 Irradiation Unit, Atomic Energy of Canada Ltd.) for incubation for five days. The control plates (**A** and **C**) were incubated at the same temperature in the absence of radiation for the same time.

Figure 11. Construction and characterization of a mercury resistant and toluene metabolizing *D. radiodurans*. **A)** MD560 is a previously constructed *D. radiodurans* strain that has the *tod* genes of *Pseudomonas putida* (Kobal *et al.*, 1973), encoding toluene

dioxygenase (TDO), cloned (Lange *et al.*, 1998) the same way as the *mer* operon in MD736 (Figure 1B). The *aphA* gene (Km^R) in MD560 was replaced with the chloramphenicol resistance gene *cat* (Cm^R) forming strain MD744. This was achieved by transformation of *Xba*I (X) linearized pMD183 (Daly *et al.*, 1994b) into MD560 with Chloramphenicol (Cm) selection. MD744 genomic DNA was then transformed into strain MD737 with double Cm and Km selection, giving strain MD764. Abbreviations and symbols are as is Figure 1. **B)** Southern blotting of genomic DNA from MD764 using both a *merA*- and a *tod*-specific radiolabeled probe. E, *Eco*RI; B, *Bam*HI; λ/H, lambda phage DNA cut with *Hind*III. **C)** 2 x 10^5 cells of R1 (*recA*$^+$, *mer*$^-$), MD737 (*recA*$^+$, *mer*$^+$), MD764 (*recA*$^+$, *mer*$^+$, *tod*$^+$) were spotted onto a TGY agar plate containing 30 μg/ml Merbromin and grown in the irradiator (Gammacell 40 Irradiation Unit, Atomic Energy of Canada Ltd.) as described in Figure 4. **D)** The production of *cis*-toluene dihydrodiol (Kobal *et al.*, 1973) from toluene by strain MD764. 1 x 10^7 cells of strain MD764 (*mer*$^+$, *tod*$^+$), pre-grown in the presence of 50 μM Merbromin, were inoculated into fresh growth medium containing 50 μM Merbromin. Toluene was introduced in the vapor phase and the cells were incubated at room temperature with shaking at 200 rpm (Gibson *et al.*, 1970). Accumulation of metabolites in the culture medium was periodically monitored by taking a one milliliter sample, removing the cells by centrifugation, and recording the UV spectra of the diluted supernatant (1:19). UV spectra of the supernatant solutions were obtained with a Beckman DU640 spectrophotometer. Strain R1 (control) was treated in an identical manner except that it was grown in the absence of Merbromin. In a separate experiment, toluene was introduced into the vapor phase of exponentially growing cells of strains MD764 (in the presence of 50 μM Merbromin) and R1 (in the absence of Merbromin). Two milliliter samples were periodically taken, the cells were removed by centrifugation, and the supernatants extracted twice with equal volumes of ethyl acetate. The organic extracts were dried over anhydrous sodium sulfate and evaporated to dryness. Each residue was re-dissolved in methylene chloride for analysis by thin layer chromatography (TLC) on silica using methylene chloride:ethyl acetate (1:1) as the solvent. The metabolites were located on the TLC plate by reacting with iodine vapor. Inset: Lane 1: *cis*-toluene dihydrodiol (Fluka Chemical); Lane 2: organic extract of strain

MD764 supernatant (20 hours); Lane 3: organic extract of strain MD764 supernatant (40 hours); Lane 4: organic extract of strain R1 supernatant (20 hours).

Figure 12. **A)** Mercuric Reductase Assay. Hg (II)-dependent NADPH oxidation catalyzed by cell extracts prepared from strains R1 (mer$^-$, tod$^-$; wildtype), MD735 (mer$^+$), MD767 (mer$^+$), MD764 (mer$^+$, tod$^+$), MD736 (mer$^+$), and MD737 (mer$^+$) were monitored spectrophotometrically according to the method of Fox and Walsh (Schottel *et al.*, 1978). The protein fractions (0.2 mg) were pre-incubated with 2 μM FAD in sodium phosphate buffer containing 2-mercaptoethanol and NADPH for ten minutes, before initiating the reaction with 0.1 mM $HgCl_2$. Decreasing Absorbance at 340 nm corresponds to a decreasing NADPH concentration. **B)** Purification of mercuric reductase. Cell extracts (30 mg protein) from strains R1, MD767, MD735, MD736, MD737 and MD764 were purified for mercuric reductase as described previously using Orange A Matrex gel chromatography (Schottel *et al.*, 1978). The protein fractions eluting with NADPH were analyzed by SDS-PAGE using an 8-25% gradient PhastGel (Pharmacia Biotech AB). Lane 1: Low Molecular Weight Range Sigmamarkers (left arrows); Lane 2, R1; Lane 3, MD767; Lane 4, MD735; Lane 5, MD736; Lane 6, MD737; Lane 7, MD764; Lane 8, low molecular weight range Sigmamarkers. Size estimates: 62 kDa; 54 kDa (right arrows). **C)** Mercury volatilization by engineered *D. radiodurans*. Strains MD735 (*mer*$^+$), MD767 (*mer*$^+$), MD736 (*mer*$^+$), MD737 (*mer*$^+$), MD764 (*mer*$^+$, *tod*$^+$), and BL308 (*E. coli*, *mer*$^+$) were pre-grown to 0.5 OD_{600} in the presence of 20 μM Merbromin, and also in 20 μM $HgCl_2$. The control strains R1 (wildtype), MD744 (*mer*$^-$, *tod*$^+$), MD560 (*mer*$^-$, *tod*$^+$) were pre-grown to the same OD_{600}, but in the absence of Hg (II). Cells of each strain were harvested, washed twice in fresh medium lacking Hg (II), concentrated to OD_{600} 2.0 in fresh medium, followed by the inoculation of 1 x 10^7 cells (~50 μl) of each into 200 μl of medium containing 30 μM $HgCl_2$ (top), or 30 μM Merbromin (bottom), contained in 300 μl wells of a microplate. Cells, pre-grown in Merbromin, were tested for Hg-volatilization in Merbromin-containing wells. Cells, pre-grown in $HgCl_2$ were tested for Hg-volatilization in $HgCl_2$-containing wells. Then, the plate was covered with a sheet of X-ray film, held together with a weight, and incubated in the dark at 32°C. Following

exposure for 14 hours, the film was developed.

Figure 13. Cold vapor atomic fluorescence spectroscopy (CVAFS). *D. radiodurans* strain MD764 was grown in TGY containing Cm plus 10 μM $HgCl_2$. Exponentially growing cells (150 ml; OD_{600} 0.6) were harvested by centrifugation and resuspended in 75 ml TGY medium containing Cm and Km, before incubation at room temperature for two hours. The cells were harvested again, washed with TGY before being resuspended in TGY to an OD_{600} of 1.8. The reaction with Hg (II) was begun by adding 25 μl of 10 mM $HgCl_2$ to 25 ml of the concentrated culture (final concentration 10 μM $HgCl_2$). Wildtype *D. radiodurans* strain R1 cells were treated identically except that the cells were pre-grown in TGY lacking Hg (II). At the times indicated, one milliliter samples were taken and added to 10 ml bromine monochloride and treated in a manner similar to that reported by Bloom and Crecelius (Bloome *et al.*, 1983). Appropriately diluted samples were reduced with stannous chloride and the resulting Hg (0) concentrated on gold-coated sand traps and analyzed by CVAFS using a Brooks-Rand Model III analyzer equipped with Mercury Guru 2.0 software. All solutions, including TGY, were prepared with ultra-pure water (Millipore Milli-Q Water System) and all laboratory glass- and plastic-ware were washed in warm 6 M HCl for at least twelve hours before use.

Figure 14. Transformation of *D. geothermalis* with an autonomously replication 26 kilobase plasmid (pMD66) designed for *D. radiodurans*.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENT

I. General Description

The most radiation resistant organism yet discovered is *Deinococcus radiodurans* (Brooks *et al.*, 1980; Minton, 1996; Daly *et al.*, 1994a). *D. radiodurans* is a non-pathogenic, desiccation resistant (Mattimore *et al.*, 1996), solvent tolerant (Lange *et al.*, 1998), soil bacterium that can survive acute exposures to ionizing radiation of 15,000 Gy without lethality or increasing mutation frequency (Daly *et al.*, 1994a); this dose induces >130 double strand breaks (DSBs) per haploid chromosome (Daly *et al.*, 1994a).

Further, this bacterium can grow continuously in the presence of 60 Gy/h (a dose rate that exceeds those at radioactive DOE waste sites [Riley *et al.*, 1992]) with no effect on either its growth rate or ability to express foreign genes (Lange *et a*l., 1998). This ability is extraordinary since most cells cannot survive more than 50-500 Gy (Thornley, 1963), or 1-3 DSBs per haploid chromosome (Krasin *et al.*, 1977). Recent advances in the ability to genetically manipulate this bacterium (Lange *et a*l., 1998, Daly *et al.*, 1994b; 1995; 1996; 1997) have led to insights into its DNA repair capabilities. The mechanism of radiation resistance has been shown to be due, in part, to exceedingly efficient *recA*-dependent (Daly *et al.*, 1994a; 1994b; 1995; 1997) as well as *recA*-independent DNA repair processes (Daly *et al.*, 1996). Likewise, *D. radiodurans* is also extraordinarily resistant to most chemical DNA damaging agents such as mitomycin-C, nitrous acid, and 4-nitroquinoline-*N*-oxide (Minton, 1996; Moseley *et al.*, 1983; Minton, 1994).

The ability of a microorganism to resist the toxic effect of metals is frequently associated with its ability to transform those metals to less toxic chemical states. Cloning metal resistance genes into *D. radiodurans*, therefore, serves two important objectives: 1) to confer resistance to the most common metallic waste constituents; and 2) to transform those metals to less toxic and less soluble chemical forms. Generally, the solubility of metals is reduced at lower oxidation states, and enzymes catalyzing such metal reducing functions are becoming important components of metal bioremediation strategies. For example, the bacterial mercuric reductase gene, *merA*, encodes mercuric ion reductase (MerA), that reduces highly toxic, thiol-reactive mercuric ion, Hg (II), to much less toxic and nearly inert monoatomic Hg (0) (Hamlett *et al.*, 1992). Ionic Hg (II) is a frequent metal contaminant at DOE facilities (Riley *et al.*, 1992; McCullough *et al.*, 1999); there may be as many as 250 DOE waste sites contaminated with Hg (II) (Riley *et al.*, 1992). Mercuric (II) ions are extremely toxic to humans and other organisms due to their avid binding to sulfhydryl groups and, therefore, inhibit many enzyme-catalyzed reactions (Creighton, 1993). While some bacteria activate Hg (II) to more toxic forms (*e.g.*, dimethylmercury), others can detoxify and remediate the ion via a reductive enzymatic reaction that produces volatile elemental mercury. Mercury (0) is relatively non-toxic to bacteria, plants, animals, and humans. The genes responsible for the reaction, most

notably the gene mercuric reductase (*merA*), are widely distributed in bacteria, and have been cloned and expressed in transgenic plants (Rugh *et al.*, 1998). MerA is a member of the flavoprotein redox-active disulfide family of proteins.

To demonstrate the applicability of the strategy to confer both metal resistance and metal remediating capabilities on a radiation resistant bacterium, the present inventors cloned the highly characterized *merA* locus from the *Escherichia coli* strain BL308 (*E. coli* K12 containing pDB7, [Barineau *et al.*, 1984]) into *D. radiodurans* (Rainey et al., 1997; White, 1999). Four different *D. radiodurans* expression systems were tested. Further, by designing bioremediating *D. radiodurans* targeted at specific, and possibly unique, radioactive sites, the present invention includes bacterial strains engineered to combine a variety of different gene-encoded functions into a single host.

One embodiment of the invention includes extremely radiation resistant *D. radiodurans* strains which express *mer*-encoded gene functions and are: 1) resistant to the bacteriocidal effects of ionic Hg (II) at concentrations (50 μM; Figure 9D) well above the highest concentration reported for mercury-contaminated DOE waste sites (10 μM [Riley *et al.*, 1992]); and 2) reduce toxic Hg (II) to much less toxic elemental and volatile Hg (0) (Figures 12, 13).

II. Specific Embodiments

Organic Toxin Degradation

While microorganisms can degrade most natural compounds, few are able to degrade synthetic compounds such as fuel hydrocarbons (*e.g.*, toluene) or halogenated hydrocarbons (*e.g.*, TCE or PCBs). The scarcity of appropriate microbial enzyme systems to degrade these serious recalcitrant hazardous pollutants (Infante *et al.*, 1982, Jacobson *et al.*, 1996) is a reflection of the relatively recent introduction of these xenobiotics to Earth. Since the introduction of these compounds into the biosphere about fifty years ago, natural evolution has begun to modify pre-existing bacterial genes to make enzymes capable of metabolizing such synthetic organic chemicals (Chakrabarty, 1996). In the last fifteen years, researchers have identified some of these genes (mostly from *Pseudomonas* spp.)

and have begun to refine their pollutant-degrading capabilities by genetic engineering (Wackett, 1997, Wackett *et al.*, 1988).

The use of *in situ* bioremediation for organic toxin-contaminated soils and groundwaters poses as a viable alternative to chemical methods that utilize costly pump and treat technologies (McKay *et al.*, 1989) and/or soil excavation and incineration. Typical organic solvents used by DOE: benzene, toluene, ethylbenzene and xylenes (collectively called BTEX), are known growth substrates for some organisms (*e.g.*, *Pseudomonas* spp.), of which the genetics and biochemistry have been studied in great detail. Furthermore, it has been discovered that co-contaminating haloorganic solvents such as TCE are biotransformed (co-oxidized) during aerobic metabolism of certain aromatic compounds (*e.g.*, toluene) where broad specificity oxygenases from toluene catabolic pathways can, typically, co-oxidize TCE. Compounds such as high molecular weight PCBs, that were originally thought to be non-degradable by microbes, are regularly being found to be transformed by bacteria utilizing biphenyl and low molecular weight PCBs for growth (Focht, 1995). With respect to DOE facilities, up until now, there has been no adequate method for microbiological treatment of contaminant waste sites containing both hazardous organic and radioactive components since organisms like *Pseudomonas* spp. are very radiation sensitive.

Table 1. Distribution of Compound Classes in Soil[5] and Groundwaters and the Number of Sites (out of 91) Containing Mixtures of those Compounds as a Function of DOE Facility[1].

	Distribution (by % of waste Sites)[2] of Compound Classes[3]					Number of Reported Compound Class Combinations				
Facility	#	A[4]	B[4]	C[4]	D[4]	A+B[4]	A+C[4]	A+D[4]	A+B+C[4]	A+B+D[4]
Argonne Nat. Lab.	2	0 *10* *0*	0 *50*	0 *0*	0 *0*	*1*				
Brookhaven Nat. Lab.	4	0 *25*	0 *10* *0*	0 *0*	0 *75*	*1*				
Fernald	11	72 *72*	45 *72*	10 0 *0*	27 *72*	3 *8*	8	1 *8*	3	1
Hanford Site	7	85 *71*	28 *42*	14 *0*	14 *0*	2 *3*	1	1	1	
Idaho Nat. Engin. Lab	6	50 *50*	33 *66*	33 *0*	16 *16*	2 *2*	2	1 *1*	2	1 *1*
Oakridge Nat. Lab.	9	33 *44*	11 *77*	33 *0*	22 *22*	1 *3*	2	1 *2*	1	1 *2*
Pantex Plant	3	0 *66*	66 *33*	0 *0*	10 0 *33*	*1*		*1*		
Rocky Flats Plant	3	66 *10* *0*	10 0 *10* *0*	0 *0*	33 *33*	2 *3*		1 *1*		1 *1*
Savannah River Plant	9	33 *77*	55 *77*	0 *0*	33 *0*	1 *6*				

[1] Data obtained from Riley *et al.*

: Number of sample waste sites evaluated at a specific facility.

[2] Percent of sampled waste sites at a specific facility reporting a specific compound class.

[3] Compound-class index:

A = **radionuclides and toxic metals**

B = **chlorinated hydrocarbons**

C = **polychlorinated biphenyls**

D = **fuel hydrocarbons**

[4] Column cell numbers refer to compound class in soil (top) and in groundwater (bottom italic).

[5] Soils and Sediments.

Many subsurface environments are anoxic and anaerobic microorganisms are being isolated from these environments that are capable of transforming pollutants. For example, toluene and benzene are known to be degraded anaerobically using alternative electron acceptors other than O_2 and TCE is dehalogenated by certain anaerobes to less halogenated ethylenes. Examples of these anaerobic bacteria include dissimilatory iron-reducing bacteria (DIRB) that can utilize ferric iron associated with aqueous or solid phases as a terminal electron acceptor coupled to the oxidation of organic substrates (Lovley, 1991). When stimulated for Fe(III) reduction, such microbes can efficiently remove hydrocarbons (*e.g.*, benzene) in anoxic environments. However, the genetics and biochemistry of anaerobic processes are, in general, poorly characterized and many of the genes encoding these activities are not yet identified/cloned (Lovley, 1995).

Even when the dissolved O_2 concentrations of groundwaters are between 0-400 μM, aerobic systems can be generally applied to contaminated groundwaters. In those environments where oxygen is limiting, bioventing and biosparging strategies have been developed to circumvent this problem. The general rule is that if there is any oxygen present, it will likely be the primary terminal electron acceptor, and aerobic processes will function, although perhaps slower at low O_2 concentrations. The use of biodegradation pathways from aerobes, such as those described herein, is not of primary concern because

the typical K_m for O_2 by those enzymes employed in such pathways is much lower than the concentrations of O_2 typically found in environments with low oxygen tensions, such as in groundwater.

Metal Toxicity Resistance

A number of biological systems have been described for resistance to heavy metals which may be used to increase the resistance of *D. radiodurans* to the toxic effects of metals and radionuclides present in DOE wastes (Riley, 1992). If *D. radiodurans* is not naturally resistant to metals over the concentration ranges that are found at DOE sites, strains of the invention can be engineered for resistance to those metals by either natural selection or by genetic engineering. For instance, many heavy metal resistance (export) systems are functional in *Alcaligenes eutrophus* CH34 (Diels *et al.*, 1995) that has multiple heavy metal resistance genes, and many modes of detoxification. These genes include: *czc* (Cd^{2+}, Zn^{2+}, Co^{2+}), *cnr* (Co^{2+}, Ni^{2+}, Zn^{2+}), and *mer* (Hg^{2+} and organomercury), as well as other genes recently cloned for resistance to Cu^{2+}, Pb^{2+} and Mn^{2+}, all of which may be cloned and expressed in *Deinococcus* as described below.

An alternative to using *A. eutrophus'* metal-exporting genes, is cloning metallothionein (MT) genes into *Deinococcus*. The Cyanobacterium *Synechococcus* produces MT-like proteins that provide resistance to the toxic effects of Zn^{2+}, Cd^{2+}, and Hg^{2+} by intracellular sequestration. In *E. coli*, expression of the *Synechococcus* genes encoding the MT-like proteins caused enhanced intracellular accumulation of Zn^{2+}, Cd^{2+} and Hg^{2+}. MT-like proteins have also been isolated from Cd^{2+}-resistant *Pseudomonas putida* (Higham *et al.*, 1984). The cloned genes encoding these low molecular weight MT-like polypeptides may be introduced into any of the *Deinococcal* or other radiation resistant strains, including those described herein. For resistance to the semi-metal arsenic, cloning the *ars*B and *ars*C As-efflux resistance genes from the Gram-positive genus *Staphylococcus* are also expressible in *Deinococcus*. The metals found most frequently associated with radionuclides at DOE sites are listed in Table 2 (the highest groundwater concentrations in 'mM' are also shown).

Table 2

Metal	Groundwater Concentration (µg/L)	Ground Concentration (µg/kg)
Lead	0.56 - 120,000 (1.1 mM)	1,000 - 6,900,000
Chromium	0.42 - 9,010 (0.17 mM)	5.1 - 3,950,000
Arsenic (semi-metal)	0.3 - 32,100 (0.4 mM)	100 - 102,000
Zinc	1 - 697,000 (10.7 mM)	150 - 5,000,000
Copper	1 - 3,300 (0.052 mM)	30 - 550,000
Cadmium	0.005 -7,600 (0.06 mM)	100 - 345,000
Mercury	0.08 - 216,900 (1.1 mM)	0.1 - 1,800,000

DOE Wastes Targeted for Bioremediation Research

The individual organic chemical constituents of radioactive wastes sites targeted for microbiological remediation are given in Table 3. On the basis of the frequency of occurrence, the following organic chemical representatives of specific compound classes may be the primary targets for the bioremediation bacteria, compositions and methods of the invention: 1) fuel hydrocarbon class: toluene; 2) chlorinated hydrocarbon class: trichloroethylene; 3) PCB class: Arochlor 1248.

Table 3. Among the 91 DOE Sites[A] Screened, Number of Selected Compound Class Constituents in Soils[B] and Groundwaters.

Compound Class	Class constituent	Ground[C]	Groundwaters[C]
Chlorinated hydrocarbons	Trichloroethylene	11	14
	1,1,1 -Trichloroethane	10	11
	Tetrachloroethylene	9	10
	Dichloroethane	9	7
	Carbon tetrachloride	6	7
	Chloroform	5	10
Fuel hydrocarbons	Toluene	8	8
	Xylene	5	8
	Ethylbenzene	5	6
	Phenanthrene	4	NR[D]
	Anthracene	4	NR[D]
PCBs	Arochlor 1248	4	0
	Arochlor 1016	1	1
	Arochlor 1242	2	2
Radionuclide/Metal	Uranium (γ, α)[E] / Lead	12 / 16	12 / 5
	Plutonium(α)[E] / Chromium	10 / 13	5 / 6
	Cesium (γ, β^-)[E] / Arsenic	10 / 13	5 / 6
	Tritium (β^-)[E] / Zinc	6 / 13	12 / 6
	Strontium (β^-)[E] / Copper	6 / 12	9 / 7
	Thorium (α)[E] / Cadmium	3 / 10	5 / 6

[A] Data obtained from ref. 81.

[B] Soils and Sediments.

[C] Number of sample waste sites (out of 91) reported at 18 DOE Facilities.

[D] Not Reported.

[E] Mode of decay.

Recombinant Bacterial Strains

The recombinant bacteria of the invention are resistant to the damaging effects of radiation. Preferred strains are resistant to acute exposure to ionizing radiation of up to about 15,000 Gy or are resistant to continuous or chronic exposure to ionizing radiation of up to about 60 Gy/hour. Bacterial species that may be engineered include species and strains of *Enterococcus*, *Alcaligenes* and *Deinococcus*, although other radiation resistant bacterial species or species of other microorganisms may be used. Radiation resistant strains of *Deinococcus* include, but are not limited to, strains of *D. radiodurans*, *D. radiopugnans*, *D. grandis*, *D. proteolyticus*, *D. murrayi*, *D. geothermalis*, and *D. radiophilus* as well as other uncharacterized *Deinococcus* isolates. Preferred bacterial strains are engineered strains of *D. radiodurans* and *D. geothermalis*.

Bacterial strains of the invention may be engineered using any available technologies, including available plasmids or vectors, selection markers, transformation systems or methods, etc. For instance, a number of autonomously replicating plasmids and chromosomal integration vectors for *D. radiodurans* are available that allow the expression of heterologous genes at high copy number (Daly et al., 1994a; 1994b; 1995; 1996; 1997)

There are at least four expression systems available for *D. radiodurans* that are summarized below. For example, the present inventors have developed a large number of shuttle vectors including integrating and plasmid vectors for use in *D. radiodurans* and *E. coli*. These vectors are used to express foreign genes in *D. radiodurans* and *D. radiodurans* genes in *E. coli*. The first *D. radiodurans* plasmids, typically, were composed of an *E. coli* plasmid containing a kanamycin resistance gene (Km^R) and some *D. radiodurans* chromosomal DNA (Smith *et al.*, 1988). In *E. coli* these plasmids replicated

autonomously, but in *D. radiodurans* they conferred Km^R by chromosomal insertion. This type of expression is summarized below as a Type-IV expression system.

The *D. radiodurans* expression vectors typically consist of an assortment of characterized DNA segments containing discrete functional elements (*e.g.*, for promoting or replicating). One class contains autonomously replicating plasmids, while the remaining three contain chromosomal integration vectors. All four vector types can be propagated in *E. coli* and subsequently used to transform *D. radiodurans* and other *Deinococcal* strains, such as *D. geothermalis*. These specialized *Deinococcus-E. coli* plasmids were tailored for optimal function and are highly characterized (Daly *et al.*, 1994a; 1994b; 1995; 1996; Smith *et al.*, 1988). Generally, expression of cloned genes in *D. radiodurans* is regulated by varying foreign gene dosage in combination with constitutive promoters.

Type-I: Autonomous plasmids: These plasmids are autonomously replicating DNA circles in *D. radiodurans* and, typically, contain two constitutive promoters; one for driving an antibiotic resistance gene, the other for driving a foreign gene. They exist at six copies per cell (Daly *et al.*, 1994a).

Type-II: Chromosomal direct-insertion vectors: Vectors of this class will integrate into the *D. radiodurans* chromosome by homologous recombination, leaving a single permanent copy per chromosome (there are 4-10 identical chromosomes per cell). Foreign genes integrated into the chromosome this way are promoted by adjacent constitutive *D. radiodurans* promoters (Daly *et al.*, 1995).

Type-III: Chromosomal duplication-insertion vectors: These vectors will integrate into the chromosome by homologous recombination leaving 10-20 transient copies per cell. Unlike a Type-II insertion, a Type-III chromosomal insertion can be lost by extended growth in the absence of any selection, restoring the original chromosomal sequence. Foreign genes located within these chromosomal insertions are expressed by a constitutive promoter (Carrol *et al.*, 1996, Daly *et al.*, 1995, Daly *et al.*, 1996).

Type-IV: Chromosomal amplification vectors: These vectors are very similar to Type-III vectors. How-ever, upon homologous integration, these vectors amplify in the chromosome yielding 80-500 vector copies per cell. Expression of foreign genes, within the amplification unit, is proportional to the number of integration copies per cell (Smith *et al.*, 1988).

The four expression systems for *Deinococcus* summarized above can be combined into the same host cell. For instance, the present inventors have constructed a number of *D. radiodurans* strains, each containing two different gene expression types marked with either resistance to kanamycin (Km^R) or chloramphenicol (Cm^R) (*e.g.*, Km^R-Type-II plus Cm^R-Type-III (Daly *et al.*, 1995); Km^R-Type-III plus Cm^R-Type-III (Daly *et al.*, 1996); Km^R-Type-I plus Cm^R-Type-III (Dowling *et al.*, 1993)). These different combinations were initially selected for in the same hosts by double drug selection and were shown to be maintained by cells even without any selection following irradiation (Daly *et al.*, 1995; Daly *et al.*, 1996; Daly *et al*, 1997). Any of these vectors may be used to engineer bacterial strains of the invention. Preferred strains may express metal resistance genes using Type-II (direct-insertion) vectors and toxin-degrading genes using Type-III and Type-IV chromosomal insertion using vectors.

Engineered bacterial strains of the invention may contain any available genes, loci or operons that encode proteins that degrade, metabolize or detoxify toxins such as organic chemicals, metals or other compounds found in waste sites. For instance, the *P. putida todC1C2BA* and *E. coli merA* operons may be cloned into the radiation resistant bacterial strain of choice. These genes may be used to augment the native ability of the recombinant strains to degrade or detoxify toxins or heavy metals. Numerous other degradatory or resistance functions from other bacteria, such as resistance functions specific for metals, may be cloned as set forth in Table 5.

Table 5 Degradative Pathway Genes or Metal Resistance Genes

Substrate	Degradative Pathway Genes / Plasmid or Metal Resistance Genes / Plasmid	Organism	Reference
toluene/TCE	*todC1C2BA* / pHG-2 cloned into *D. radiodurans*	*Pseudomonas*	111
toluene/TCE	Tol region / pDKR1; pRP1	*Pseudomonas*	113
toluene	*xylL-xylE* / pBK187	*Pseudomonas*	107
toluene	upper and lower TOL operons / pWW53-4, pEHK11, pEHK355	*Pseudomonas*	53
toluene	monooxygenases: / pMS64, PK01, KR1	*Pseudomonas*	52,73, 80, 89
TCE	gene clusters involving *bphA1A2A3A4* and *todC1C2BA* / pJHF3051, pJHF301 and pJHF108	*Pseudomonas*	32
PCBs	*bph* gene operon subclones/ pDD5301, pDD530, pDD5201, pDD5211	*Pseudomonas*	2, 8, 21
Cd, Zn, Co, Hg, Mn, Pb	*czc*C, *czc*B, *czc*A pMOL30	*Alcaligenes eutrophus, Ralstonia eutrohus*	20
Zn, Cd, Hg	*smt*A, *smt*B	*Synechococcus*	102
As (semi-metal)	*ars*A, *ars*B	*Staphylococcus*	50, 86
U(VI)	*cytc*3	*Desulfovibrio vulgaris*	
Cr(VI)		*Bacillus thuringiensis*	

Growth Media

The present inventors have also developed a synthetic minimal media which may be used to engineer strains of the present invention and to practice the claimed methods. In developing a synthetic minimal medium, many combinations of varying amounts of carbohydrates, amino acids, salts and vitamins in both liquid and solid medium were systematically tested. By a process of elimination, minimal nutrient constituents, and their concentrations, necessary for luxuriant growth were identified as set forth in Table 5. This synthetic medium preparation is distinct in that it is much simpler, and growth of *D.*

radiodurans in such medium is completely dependent on a carbon/energy source. In addition to a metabolizable carbon source, growth of *D. radiodurans* is dependent on exogenous amino acids and a vitamin; addition of the sulfur-rich amino acids cysteine and histidine, together with nicotinamide were particularly effective at supporting growth. However, the specificity of amino acids was shown is not stringent in that many different combinations of amino acids support growth. A factor that strongly influences the extent of growth is the total amino acid concentration in the growth medium, and not the composition of the amino acid pool. Among the carbon sources tested, the following supported luxuriant to slow growth in the following order: fructose > pyruvate > lactate > glucose > oxaloacetate > acetate > glycerol.

Table 5. *D. radiodurans* minimal nutrient requirements for growth in the absence (A) and presence (B) of γ-radiation

	Compounds:	Concentrations: A / B
BSM:		
	Potassium Phosphate Buffer (pH7.5-8.0)	20 mM / 20 mM
Salts:		
	Magnesium Chloride, Tetrahydrate	0.2 mM / 0.2 mM
	Calcium Chloride, Dihydrate	0.1 mM / 0.1 mM
	Manganese(II) Acetate, Tetrahydrate	5.0 μM / 5.0 μM
	Ammonium Molybdate, Tetrahydrate	5.0 μM / 5.0 μM
	Ferrous Sulfate, Heptahydrate	5.0 μM / 5.0 μM
Amino Acids:		
	Histidine	25 μg/ml / **2.5 mg/ml**
	Cysteine	25 μg/ml / **2.5 mg/ml**
Vitamin:		
	Nicotinic Acid	1.0 μg/ml / 1.0 μg/ml
Carbon:		
	Carbon Source	2 mg/ml / 2mg/ml

Basal Salt Medium (BSM) was autoclaved and then supplemented with sterile

preparations of salts, amino acids and nicotinamide, to the indicated concentrations. For solid medium, Nobel Bacto Agar was added before autoclaving BSM, to 1.5% (w/v). Individual carbon sources were added to a concentration of no more than 2 mg/ml. The concentrations shown on the left (**A**) are those used for growth in the absence of radiation. The concentrations shown in bold on the right (**B**) are changes made to nutrient conditions shown in A that supported growth in the presence of continuous radiation (60 Gy/hour). Growth media for continuous radiation exposure may also be suplemented with other amino acids at the following approximate concentrations: glutamine, 500 µg/ml; alanine, 500 µg/ml; arginine, 800 µg/ml; asparagine, 800 µg/ml; glycine 300 µg/ml; leucine, 500 µg/ml; lysine, 300 µg/ml; methionine, 100 µg/ml; proline, 370 µg/ml; serine 300 µg/ml; threonin, 200 µg/ml; tryptophan, 200 µg/ml; tyrosine, 200 µg/ml; and valine, 200 µg/ml. Substitution of Nicotinic acid with Basal Medium Eagle Vitamin Solution (GibcoBRL) improves growth slightly.

Contaminated Waste

As used herein, the term "toxins" includes organic, radionuclide and inorganic or metallic contaminants, as well as non-petroleum organic contaminants, particularly those found in industrial waste, waste generated from the production of nuclear weapons and waste produced from the civilian uses of radionuclides. Such contaminants are also often found in waste generated from textile and paper mills, chemical manufacturers, and transportation facilities, as well as restaurants and institutions, such as commercial kitchens, food processing plants, and the like. Other sources of contaminant production include crude oil spills, chemical and solvent leaks, fuel oil leaks, and creosote contamination.

As used herein, inorganic contaminants include the contaminants described above as well as inorganic sulfur and ferrous compounds, metallic elements, such chromium, lead, arsenic, zinc, cadmium, cobalt, mercury and certain copper compounds used as herbicides and algicides.

Organic contaminants include the contaminants described above as well as various pesticides, such as insecticides, growth regulators, growth inhibitors, toxicants, bactericides, attractants, repellants, hormones, molluscicides, defoliants, chemosterilants, fumigants, systemics, rodenticides, avicides, detergents, surfactants, nematicides,

acaricides, miticides, predicides, herbicides, agricultural chemicals, algicides, fungicides, sterilants; polycyclic aromatic hydrocarbons (PAH's), polychlorinated biphenyls (PCB's), greasy wastes, solvents, crude oil, diesel fuel, waste oil, Bunker "C" oil, phenolics, halogenated hydrocarbons, citrus juice processing wastes, terpene alcohols, starchy carbohydrates, and the like.

Examples of specific organic contaminants include those described above as well as anthracene, chlorotoluenes, chrysene, cresols, di-N-octylphthalate, dichlorobenzene, dichlorethanes, dichloropropanes, dichlorotoluene, 2-ethoxyethanol, ethylene glycol, ethylene glycol monoethyl ether acetate, ethylbenzene, fluorene, isoprenoids, methyl ethyl ketone, methylene chloride, naphthalene, pentachlorophenol, phenanthrene, 1,1,2,2-tetrachloroethane, toluene, 1,1,2-trichloroethane, trichloroethylene, benzoate, chlorobenzoates, methanol, ethyl acetate, cyclohexanone, ethylbenzene, 2,4-dichlorophenoxyacetic acid, 2,4,5-trichlorophenoxyacetic acid, m,o,p-xylene, butyl acetate, camphor, hexane, heptane, octane, nonane, d-limonene, linalool, geraniol and citronellol.

Bioremediation compositions

Bioremediation compositions of the invention may be engineered and formulated to meet the applicable regulatory requirements, including the requirements of the Department of Energy as outlined in McCullough *et al.*, Bioremediation of Metals and Radionuclides, which is herein incorporated by reference in its entirety. For instance, bioremediation compositions may include film-forming agents and/or nutrient agents in additions to the bacterial strains of the invention. These can be used singly or in various combinations. In a preferred embodiment for use in aqueous environments or clean-up sites, bioremediation compositions may comprise at least one film-forming agent. The use of a film-forming agent in combination with a bacterial strain of the invention generally acts to enhance the activity of the bacterial strain. For example, film-forming agents can be used to increase the surface area where oil is a major contaminant by uniformly spreading a bacterial strain of the invention throughout a thin layer of dispersed oil, thereby accelerating the biodegradation process. Not only is the oil made more readily

available to the bacterial strain, but mixing and dilution of emulsion droplets in a greater volume of water assures a more adequate supply of nutrients for the bacterial strain utilized.

Film-Forming Agents

As used herein, the term "film-forming agent" is meant to include dispersants, surface-active agents, surfactants, detergents, and the like. Typically, this class of chemicals have an oil-soluble end (*i.e.*, a hydrocarbon chain) and a water soluble end (*i.e.*, polar groups, such as carboxylate, sulfonate, ether, alcohol, or polyethylene oxide). Because of this dual nature, film-forming agents orient at the surface contaminant/water interface. Surface contaminants refer to contaminants which have a tendency to float on the surface of water. Specific examples of surface contaminants include oil or petroleum. When applied to aqueous waste contaminated with oil, for example, a film-forming agent will reduce the surface tension of the water while spontaneously and rapidly spreading over the surface of the water to form a near monomolecular or duplex film that can push or concentrate oil or other surface contaminants into a confined area for clean-up. Alternatively, when applied to oil, the oriented film-forming molecules can also reduce the interfacial tension between the oil and water thereby "weakening" and reducing the cohesiveness of the oil slick. Additionally, the hydrophilic groups of the film-forming molecules on the surface of the oil droplets repel other droplets and prevent coalescence. The hydrophilic surface also reduces the tendency of the droplets to stick to solid.

Film-forming agents that are suitable for use in the present invention are generally more oil soluble than water soluble and preferably are only minimally water soluble. Furthermore, film-forming agents suitable for use in the present invention are organic materials which spread rapidly and spontaneously into extremely thin films approaching monomolecular dimensions. Consequently, small quantities of film-forming agents will affect large areas of a water surface. These film-forming agents are generally autophobic, nonionic, nonvolatile organic liquids with a density less than water. Typically, they have a low freezing point and a boiling point above the maximum air temperature of the environment into which they are placed. The freezing point can be below about 5°C. The

boiling point can be about 170°C or higher, preferably it is at least about 200°C. These film-forming agents have an HLB (Hydrophile Lipophile Balance) number of 10 or less, a bulk viscosity of less than 1000 centistokes at the temperature of use, a surface tension effectiveness which lowers the surface tension to approximately 35 dynes/cm or less, and are generally capable of rapidly and spontaneously spreading with high spreading potentials.

Suitable film-forming agents include, but are not limited to, POE-2-isostearyl alcohol, sorbitan monooleate, sorbitan trioleate, sorbitan monolaurate, oxyethylated oleyl alcohol having two oxyethylene groups, diethylene glycol monolaurate, oxyethylated lauryl alcohol having four oxyethylene groups, an oxyethylated branched alkanol of 15-19 carbon atoms, unsaturated cis-alkanol of 12-18 carbon atoms and up to five oxyethylene groups, and an unsaturated cis-alkanol of 15-19 carbon atoms. These film-forming agents can be used in combination with an alcohol such as 2-ethyl butanol, for example. Most preferably, the film-forming agent is selected from the group consisting of POE-2-isostearyl alcohol, 65% sorbitan monolaurate and 35% 2-ethyl butanol, and 75% orbitan monooleate and 25% 2-ethyl butanol.

Nutrient Agents

Bioremediation compositions in accordance with the invention can also include nutrient agents. As used herein, the term "nutrient agent" is defined as any substance that accelerates degradation by stimulating the growth of a bacterial strain of the invention. Nutrient agents can be composed of macronutrients, micronutrients, or mixtures of both. Generally, the nutrient agents include carbon sources, nitrogen sources, phosphorous sources, or mixtures thereof. Examples of specific nutrient agents that can be used in accordance with the invention are the nutrients described in Table 4 as well as the BI-CHEM ACCELERATOR series (available from Sybron Biochemicals Inc., Birmingham NJ). The nutrient agent employed will vary according to the particular bacterial strain being used to control contaminants, as well as the environmental context of its application.

One embodiment of the invention includes the use of nutrient agents to enhance the

activity of the bacterial strain. In some instances, the nutrient agent and the bacterial strain are both impregnated within a polymer to enhance bioremedial activity.

Controlled Release Contaminant-Reducing Agent Delivery Compositions

The bioremediating compositions of the present invention can be prepared by mixing, encapsulating, agglomerating, or formulating one or more bacterial strains of the invention with one or more non-toxic and inert adjuvants or diluents into compositions such as solid powders, dusts, granules, pellets, briquets, extrusions, laminates, or composites, or into sprayable, pumpable, or injectable, variable-viscosity water or oil-base formulations such as gels or semi-gels. These compositions can be optionally incorporated into water-soluble or biodegradable/degradable packets, pouches, or capsules, made of, for example, polyvinyl alcohol, hydroxypropyl methyl cellulose, polyethylene oxide, or gelatin, or insoluble devices made, for example, of polyethylene or polypropylene, for use as secondary delivery vehicles for contaminant-reducing compositions.

In particular, the present invention is directed toward a method of formulating one or more bacterial strains of the invention, with or without water or other additives, into compositions such as solid powders, dusts, granules, agglomerates, pellets, briquets, extrusions, laminates, or composites, or into sprayable, pumpable, or injectable, variable-viscosity water or oil base gel or semi-gel like formulations that can release one or more active ingredients to simultaneously or concurrently control a variety of inorganic or organic contaminants with a single or multiple application of a solid or liquid single or multi-product formulation. Preferably, the release occurs in a controlled manner.

The slow or controlled release process may be modified or delayed by the degree of compaction of the formulation, by varying the size of an orifice or the number of orifices in a container into which the formulation is placed, by varying the concentration of film-forming agent, by varying the concentration of different types of polymers, and by adding one or more binders. For example, using one or more cationic, anionic, or nonionic surfactants or surface active agents in the composition can regulate the rate and duration of delivery (*i.e.*, increase or decrease).

Preferably, the bioremediating compositions of the present invention contain one or more nontoxic and inert adjuvants or diluents such as carriers, binders, coatings, defloculating agents, penetrants, spreading agents, surface-active agents, surfactants, suspending agents, wetting agents, stabilizing agents, compatibility agents, sticking agents, waxes, oils, co-solvents, coupling agents, foams, antifoaming agents, synthetic plastics, elastomers, synergists, natural or synthetic polymers, UV protectants, buoyancy modifying agents, biocides, and other additives and mixtures thereof. Some materials may be biodegradable, or photodegradable (*e.g.*, ultraviolet light), and others may be degraded by hydrolysis.

Bioremediating compositions of the invention can be applied to the contaminated site by conventional ground, aquatic or aerial techniques as outlined by McCullough *et al.*, 1999. In a terrestrial environment, the composition can be applied directly on the soil surface, introduced into one or more sub-surface layers, mulched into the soil, introduced into biopiles or prepared beds or composted with contaminated soil or materials. The methods of the invention may also include the use of bioreactors and other bacterial growth augmentations methods.

In an aquatic environment, the composition may be applied to uniformly mix within the aquatic environment or be applied at or near the surface of water. Slurry bioreactors and sediment washing equipment may also be used in the methods of the invention. When applied directly to a contaminated water source, the bioremediating composition can be applied at a total bulk application rate of about 0.1 to about 2000 pounds per surface acre of the target environment. More preferably, the bioremediating composition is applied at a total bulk application rate of about 0.1 to about 500 pounds per surface acre of the target environment. The application range will depend upon the type of agent used, any polymers employed, the duration and rate of release desired, the total application rate required to uniformly treat the area of contamination, the type and concentration of contaminant, and the concentration of natural contaminant-reducing organisms and nutrients in the target habitat.

Without further description, it is believed that one of ordinary skill in the art can, using the preceding description and the following illustrative examples, make and utilize

the strains and bioremediation compositions of the present invention and practice the claimed methods. The following working examples therefore, specifically point out preferred embodiments of the present invention, and are not to be construed as limiting in any way the remainder of the disclosure.

EXAMPLES

Methods

The following methods were used as described below:

Growth of Cells: *D. radiodurans* and *E. coli* strains were grown in TGY medium and Luria-Bertani (LB) medium, respectively, with aeration on rotary shakers at 32°C and 37°C, respectively. Kanamycin was used at a concentration of 8 μg/ml for recombinant *D. radiodurans* strains.

Strain Construction: The regional chromosomal maps and functions of *D. radiodurans* strains MD417 and MD560 are shown in Figure 1. The tandem duplication vector pMD417 and *D. radiodurans* control strain MD417 (lacking *tod* genes) were constructed and described previously. Strain MD560 is identical to strain MD417 except for the presence of the *todC1C2BA* genes. An *Eco*RI-*Bam*HI (4.2 kb) fragment containing the *todC1C2BA* genes (Zylstra *et al.*, 1989) was cloned from plasmid pHG2 (Wackett *et al.*, 1994) into pMD417 (Figure 1) forming plasmid pMD532. MD560 is the product of transformation of wildtype strain R1 with pMD532 followed by selection on TGY plates containing kanamycin (Results). pMD532 cannot replicate as a plasmid in *D. radiodurans* because of the absence of a deinococcal plasmid origin of replication. Upon transformation, integration of pMD532 into the chromosomal target sequence BC (checkered segments, Figure 1) occurs by homologous recombination (a single cross-over) between the BC regions of the plasmid and the chromosome, respectively. As a result, the integrated vector becomes flanked on both sides by chromosomal BC sequences, forming a chromosomal tandem duplication. In *D. radiodurans*, chromosomal region 560 can confer Km^R (resistance to kanamycin encoded by a portion of the *E. coli* plasmid pMK20 [diagonally hatched region, Figure 1] that contains the *aphA* gene). Transcription of the *aphA* gene is driven by *Deinococcal* constitutive promoting sequences in a fragment

derived from the *D. radiodurans* SARK natural plasmid pUE11(black region, Figure 1). Transcription of the TDO genes in strain MD560 are driven by deinococcal constitutive promoting sequences in a fragment derived from the *D. radiodurans* SARK natural plasmid pUE10[5] (light grey region, Figure 1).

Analysis of Substrate Degradation: Toluene, chlorobenzene, 3,4-dichloro-1-butene, and TCE degradation studies were measured initially in eleven milliliter sealed reaction vials with one milliliter of concentrated cells at about 1 x 10^9 cells/ml and 25 nmol of substrate. At timed points over a one hour period, 50 ml of headspace was removed from reaction mixtures with a gas tight syringe and analyzed on a Hewlett Packard 5890 GC with flame ionization detector using a DB-1 capillary column (0.25 mm ID, 0.25 mM film thickness, 30 m length) operating at an isothermal oven temperature of 180°C, splitless injection at a temperature of 250 °C, and peak integration. The toluene and chlorobenzene *cis*-dihydrodiols were extracted from culture supernatants with ethyl acetate and analyzed by thin layer chromatography with ethyl acetate as solvent and by gas chromatography-mass spectrometry analysis using a Hewlett Packard 6890 GC with mass selective detector and Chemstation. All data were consistent with previous reported values. Further, identical products were formed with MD560 cell incubations using unconcentrated cells (OD_{600} 0.8-1.2) over a period of 12 hours (data not shown). ^{14}C-TCE experiments were conducted in sealed eleven milliliter vials using strains MD560, MD417, and a TGY control, to which 1 µCi, 20 µl of ^{14}C-TCE (8.5 mM in DMF, specific activity 6 µCi/mmole), was added each to one milliliter of cells at a density of 1 X 10^8 cells/ml. A zero time point and 18 hour time point were taken by removing 20 µl of mixture and applying the 20 µl to a 1 cm x 1 cm silica TLC plate to dry. After air drying, the TLC plates were added to five milliliter scintillation cocktail and residual nonvolatile ^{14}C measured.

For detection of indole oxidation, strains were grown to log phase in 100 ml of TGY and then incubated overnight with 100 mg of indole. Following incubation, cells were removed and the supernatants extracted twice with an equal volume of ethyl acetate. The ethyl acetate was evaporated *in vacuo* to a final volume of 5 ml, and 50 µl spotted

onto a silica thin layer chromatography (TLC) plate. Separation by TLC was carried out using ethyl acetate as the mobile phase. Commercial indigo, as well as indigo produced from *E. coli* (pDTG601a) incubations with indole, were used as controls.

Growth of D. radiodurans and Expression of TDO in the Presence of Radiation: Strains MD560 and MD417 were grown in the presence of continuous γ-irradiation (60 Gy/hr) in a ^{137}Cs Gammacell 40 irradiation unit (Atomic Energy of Canada Ltd.) at room temperature (22°C). *E. coli* was used as a negative growth control for these experiments. Survival rates were determined by plating appropriate dilutions of irradiated cells and counting the colony forming units on plates. Strains MD560 and MD417 were grown in the irradiation unit to an OD_{600} of 1.0 (1 x 10^8 cells/ml) and the cells were removed temporarily from the irradiator and concentrated to an OD_{600} of 5.0 (5 x 10^8 cells/ml). One ml of concentrated cells was aliquoted to each of five eleven milliliter vials and then 125 nmole of chlorobenzene added. Following addition of substrate, the vials were immediately placed back into the irradiator for incubation. One vial for each was removed from the irradiator at 0, 20, 40, 60, and 120 minutes after addition of substrate. Promptly following removal from the irradiator, 0.5 ml of ethyl acetate was added, the sample vigorously shaken, and frozen at -70°C. While the aqueous portion was still frozen, the ethyl acetate fraction was removed, dried with anhydrous sodium sulfate and 1 µl was analyzed by GC.

Resistance of D. radiodurans to Toluene and TCE: *D. radiodurans* strains R1, MD560 and MD417 were grown overnight in liquid growth medium and then subcultured in duplicate to an OD_{600} of 0.02 in fresh medium with varying amounts of toluene or TCE added to each. After 18 hours incubation, the cell densities were determined and plotted as a function of solvent concentration.

DNA manipulation: DNA cloning, preparation and transformations were as described previously (Daly *et al.*, 1994a; 1995; 1996; Sambrook *et al.*, 1989).

Example 1

Production of a D. radiodurans strain which expresses toluene dioxygenase

Sequence Analysis of the D. radiodurans Genome: The nearly completed *D.*

radiodurans genomic DNA sequence (<ftp://ftp.tigr.org/pub/data/d_radiodurans/>) was searched for similarity to TDO sequences using the Basic Local Alignment Search Tool (BLAST). No *D. radiodurans* sequences were found to have significant homology at the DNA level nor at the translated peptide level, suggesting that a TDO homolog does not exist in *D. radiodurans* strain R1 (wildtype).

Construction of D. radiodurans Strains MD560 and MD417: An *Eco*RI-*Bam*HI (4.2 kb) fragment containing the *todC1C2BA* genes (Zylstra *et al.*, 1989) was cloned from plasmid pHG2 (Wackett *et al.*, 1994) into the previously constructed *D. radiodurans* chromosomal tandem duplication vector pMD417 (Daly *et al.*, 1996) (Figure 1 and Experimental protocol) forming plasmid pMD532. pMD417 contains a single *Eco*RI and BamHI site in the *tet* gene. By cloning *todC1C2BA* into the *Eco*RI-*Bam*HI sites of pMD417, the *tod* genes were placed under the control of a constitutive *D. radiodurans* promoter (Figure 1 and Experimental protocol). pMD532 was transformed into *D. radiodurans* R1 followed by selection on TGY plates (Daly *et al.*, 1994a) containing kanamycin. Strain MD560 was selected and the restriction map of its chromosomal integration site (Figure 1) was confirmed by Southern analysis (data not shown). The *tod* genes were present at about two copies per chromosome (8-20 copies per cell; *D. radiodurans* has 4-10 identical chromosomal copies per cell (Minton, 1994).

Expression of TDO in D. radiodurans: The *todC1C2BA* genes cloned into *D. radiodurans* (strain MD560) are constitutively expressed to make functional TDO. *D.* radiodurans strains R1 (wildtype), MD417 (*tod*$^-$) and MD560 (*tod*$^+$) were incubated with indole (Ensley *et al.*, 1983) and only strain MD560 yielded indigo. Incubation of 1.5×10^9 cells/ml of strain MD560 resulted in complete degradation of 25 nmole/ml of toluene and chlorobenzene in thirty minutes and at near equal rates (not shown). A similar reaction with 25 nmole/ml 3,4-dichloro-1-butene resulted in oxidation of about 40% of the substrate in eighty minutes. Incubations of overnight grown cultures of MD560 (1×10^8 cells/ml) with chlorobenzene, toluene and 3,4-dichloro-1-butene yielded the anticipated diol products, as determined by GC/MS analysis of culture supernatant extracts (see Experimental Protocol). All of the *in vivo* products were identical by GC/MS analysis to

products obtained *in vitro* using purified toluene dioxygenase (data not shown). Although chlorobenzene, toluene, and 3,4-dichloro-1-butene were detectably oxidized by headspace analysis, 25 nmole/ml trichloroethylene (TCE), a substrate known to inactivate TDO (Lange *et al.*, 1997; Wackett *et al.*, 1989) was not detectably oxidized under these conditions. However, incubation of MD560 with volatile ^{14}C-TCE yielded a detectable increase in ^{14}C-nonvolatile material that was associated with the cells (not shown). This was consistent with previous studies *in vivo* (Wackett *et al.*, 1989) and *in vitro* (Li *et al.*, 1992) in which ^{14}C-TCE oxidation inactivates TDO and becomes covalently attached to cell materials. Strain MD417, lacking *tod* genes, was uniformly negative in the metabolism of all the TDO substrates tested.

Growth of D. radiodurans Strains and Expression of TDO in the Presence of Radiation: Over a period of thirty hours, *D. radiodurans*' growth characteristics and viability were not affected by the continual presence of 60 Gy/hr radiation in a ^{137}Cs irradiator (Figure 2). This level of continuous radiation exceeds those commonly found at waste sites (Riley *et al.*, 1992). *D. radiodurans* strains reached the stationary phase of their growth irrespective of the presence or absence of γ-irradiation. By comparison, *E. coli* did not grow and was killed by this level of radiation exposure, as expected. To test the ability of strain MD560 to functionally express TDO under irradiating conditions, strains MD560 and MD417 were grown in the irradiator (60 Gy/hour) for thirty hours to a cell density of 1.0 x 10^8 cells/ml. Following growth of both strains in the irradiator, each was concentrated on ice to 5 x 10^8 cells/ml and then incubated with 125 nmole/ml chlorobenzene in the presence and absence of radiation (60 Gy/hour, see Experimental Protocol). Strain MD560 oxidized 125 nmole/ml of chlorobenzene within one hour, irrespective of the presence or absence of radiation (Figure 3). Irradiator-grown control strain MD417, lacking the *tod* genes, was unable to degrade the chlorobenzene. The difference in rates observed for irradiated cells versus non-irradiated cell controls is an artifact and due to the way in which the experiment had to be conducted. The ^{137}Cs irradiator used in the experiment does not have a temperature control system and the irradiation experiments were, therefore, static and done at ambient room temperature (~22°C), whereas the non-irradiated controls were incubated in a 32°C incubator with

shaking. This resulted in an app. it time-lag within the irradiator during which the cells warmed and the poorly-soluble substrate became uniformly mixed.

Resistance of D. radiodurans to toluene and TCE: The effects of solvent concentration on growth of *D. radiodurans* strains R1, MD417 and MD560 was te

The growth of *D. radiodurans* strains was not affected up to 800 mg/L for toluene up to 1,500 mg/L TCE. These levels are well above those reported at sites (Riley *et al.*, 1992) containing contaminated groundwaters and many of those containing contaminated soil (Figure 4).

TDO was chosen for expression in *D. radiodurans* because it is prototypic of a large class of bacterial dioxygenases and has a broad substrate range that includes compounds present at sites containing organic and radioactive mixed wastes. Furthermore, TDO is comprised of four protein components with their attendant metal and organic cofactors (Wackett, 1990) and, thus, its successful expression in *D. radiodurans* indicates that many less complex biodegradative enzyme systems can be expressed.

Strain MD560, expressing TDO, oxidized indole, toluene, chlorobenzene, and 3,4-dichloro-1-butene, all known substrates for TDO. *D. radiodurans* strains grew under continuous irradiating conditions of 60 Gy/hr in a ^{137}Cs irradiator (Figure 2). Furthermore, strain MD560 synthesized functional TDO under those conditions (Figure 3) and degraded 125 nmole/ml chlorobenzene while being exposed to radiation.

The cell envelope of *D. radiodurans* includes an outer and inner lipid membrane that surrounds the cell wall (Thompson et al., 1982a; 1982b). The results presented herein indicate that the membrane architecture of this organism does not result in extreme sensitivity to organic solvents. Organic solvents are generally toxic to bacteria by making their membranes porous (deSmet *et al.*, 1978; Sikkema *et al.*, 1995). Toluene and TCE are two of the most common organopollutants at radioactive DOE waste sites (Riley *et al.*, 1992); toluene has been reported as high as 26 mg/L groundwater and 2,000 mg/kg soil, and TCE as high as 1,000 mg/L and 12,000 mg/kg. *D. radiodurans* strains R1, MD560 and MD417 were all found to be naturally tolerant to toluene and TCE groundwater concentrations well above those found at most sites, and resistant to about half the highest toluene concentrations reported in contaminated soils (Figure 4).

The lack of TCE oxidation beyond that measured using sensitive ^{14}C assay has been attributed to turnover-dependent TDO inactivation (Wackett *et al.*, 1989). Enzymatic TCE oxidation is known to generate reactive acyl chlorides that bind covalently to proteins and other macromolecules (Li *et al.*, 1992). Sustained biological TCE oxidation may, thus, can be accomplished by the intracellular biosynthesis of a scavenging nucleophile, such as glutathione, to protect against enzyme inactivation. Our analysis of genome sequences failed to detect DNA homologues to *E. coli* genes *gshA* and *gshB* (not shown). Accordingly, these genes can be cloned and expressed in *D. radiodurans* or other strains of the invention.

Example 2

Production of a D. radiodurans strain which expresses the *mer* operon

Construction and characterization of mercury (II) resistant D. radiodurans strains: The cloned *mer* operon encodes six proteins that confer mercury resistance functions on *E. coli* (Hamlett *et al.*, 1992) (Figure 7, top). The entire *D. radiodurans* genomic DNA sequence (White, 1999) was searched for similarity to these *mer* operon sequences using the Basic Local Alignment Search Tool (BLAST) (Altschul *et al.*, 1997). No authentic *mer* operon was identified in the *D. radiodurans* genome. Moreover, no orthologs of the *mer* genes were found, except *merR*- and *merD*-like genes, that are transcriptional regulators encoded in all known bacterial genomes (Schiering *et al.*, 1991). This *D. radiodurans* genomic analysis is supported by our experimental results showing the sensitivity of wildtype *D. radiodurans* strain R1 to mercury.

Strain MD735: The *mer* operon was cloned into the *D. radiodurans* autonomously replicating plasmid pMD66 (Daly *et al.*, 1994a) forming MD727, and transformed into strain R1 (MD735, Figure 7A). Briefly, *D. radiodurans* and *E. coli* strains were grown in TGY medium and Luria-Bertani (LB) medium, respectively, with aeration on rotary shakers at 32°C and 37 °C, respectively. Kanamycin (Km) and chloramphenicol (Cm) were used at a concentration of 8 μg/ml and 3 μg/ml, respectively, for recombinant *D. radiodurans* strains. Freshly prepared Merbromin (mercurochrome; 2',7'-Dibromo-5'[hydroxymercuri]-fluorescein) was used in the following concentrations, for growth on

solid medium, 30 μg/ml; in liquid medium, 50 μg/ml. Merbromin was used in our Hg (II) studies, over $HgCl_2$ because its red color (Figure 2B) allowed us to track highly toxic Hg (II) waste more easily. This construction placed the *mer* genes under the control of a constitutive *D. radiodurans* promoter (P2, Figure 7A), and Southern analysis showed that the *mer* operon was present at about one copy per cell (Figures 9A, 9B).

Strain MD736: This strain has the *mer* operon integrated into the previously described chromosomal S11 locus (Smith *et al.*, 1988), located on the 2.8 Megabase pair (Mbp) chromosome (Chromosome I [White, 1999]) of *D. radiodurans* (position 1,677,743 - 1,689,109), as a tandem duplication. The functional difference between the *mer*-containing tandem duplication vector and the *mer*-containing autonomous replicating plasmid is that the plasmid origin of replication segment (dORI; Figure 7A) was replaced with a 4 kb internal segment of the *D. radiodurans* chromosomal S11 locus, **bc** (Figure 7B). This 4 kb chromosomal segment allows it to recombine into the targeted S11 chromosome sequence (**bc**, Figure 7B) by a single crossover; without integration, a vector lacking a dORI sequence cannot replicate in the cell and is lost. Upon integration, the *mer* operon within this tandem duplication became flanked by 4 kb **bc** repeats. In strain MD736, the *mer* operon was present at about ten copies per cell (Figure 9A, 9B). It should be noted that the presence of the 18 kb *Eco*RI fragment (Figure 7B) visualized by Southern Blotting (Figure 9B) supports the presence of at least two copies on a chromosome. The fact that we detected only about ten copies per cell indicates that the strain is not homozygous; under the described culture conditions (Figure 7B), some of the 8-10 haploid copies per cell (Hansen, 1978) of Chromosome I apparently lack this insertion.

Strain MD737: Whereas the tandem duplication strain (MD736) has about ten *mer* copies per cell, amplification vectors like pS11 (Smith *et al.* 1988), integrate themselves at 150-200 vector copies per cell. When integrated, the duplicated chromosomal flanking sequences of pS11 (11.4 kb) (**abcd**, Figure 7C), can readily recombine with identical insertions on other chromosomes, leading to amplification - presumably by uneven homologous recombination of daughter chromosomes (Smith *et al.* 1988). The salient functional difference between a duplication vector (*e.g.*, in MD736) and an amplification vector (*e.g.*, in MD737) is that an amplification vector lacks a

deinococcal constitutive promoter (P1, Figure 7B) upstream of the antibiotic resistance marker (Km^R, Figure 6C). Without such a promoter, the only cells able to grow under selective antibiotic conditions are those that have highly amplified antibiotic resistance determinants (Smith *et al.* 1988). The situation in MD737 is analogous because specific transcription signals upstream of both the Km^R resistance gene (*aphA*) and the *mer* operon are lacking (Figure 7C), leading to amplification with selection pressure (Figures 7C, 9A, 9B). In strain MD737, the multiple copies of Chromosome I each contain an average of about 15 copies of the *mer* operon (~150 copies per cell) (Figures 9A, 9B).

Strain MD767: Unlike the tandem duplication and amplification vectors, a vector integrated into a host cell's chromosome by direct-insertion becomes a permanent and unchanging fixture in the cell's genome. The permanence of this integration arrangement is a result of the unique chromosomal DNA sequences flanking the integrated vector, that will not recombine (ABC and DEF, Figure 8A). This system of integration is not versatile like a tandem duplication or amplification integration since the number of integrated copies cannot be altered independently of the chromosome number. This inflexibility is a potential disadvantage for recombinant cells containing a direct insertion since they are less able to adapt to changing environmental conditions. However, the potential advantage in using this cloning approach is the fact that the insertion cannot be lost, even in the absence of any selection. And, from an environmental release standpoint, direct insertions are less likely to be transferred to other indigenous microorganisms.

Direct insertions of a plasmid in *D. radiodurans* Chromosome I have previously been constructed (Daly *et al.*, 1995). The construction of plasmids suitable for this sort of integration, however, is very labor-intensive (Daly *et al.*, 1995). A simple alternative approach to constructing a *D. radiodurans* strain containing a direct insertion of the *mer* operon was to use a genetic technique that takes advantage of this organism's transformation and recombination capabilities, and the common structural backbone of our transforming vectors (see Figure 8). This is the first demonstration in *D. radiodurans* showing how a gene cloned as a duplication insertion can be converted to a direct (permanent) insertion by recombinative transformation. Following selection for mercury resistance (Figure 8B), individual clones were pre-screened for resistance to

chloramphenicol to select for crossovers in the desired chromosomal regions, yielding strain MD767 (Figure 7C), that contains one *mer* operon per 2.8 Mbp-chromosome (Figures 8A, 8B).

mer copy number: The copy number of the *mer* operon in each of the engineered strains (MD735, MD736, MD737, and MD767) was determined (Figures 9A, 9B; the agarose gel is aligned with the Southern blot). The *merA* copy number in these four strains, growing exponentially, was determined by comparing the number of radioactive disintegrations of each hybridizing band to the *merA*-specific band of strain MD767 (Figure 9B), that contains about ten copies of the *mer* operon per cell; the direct insertion is located on Chromosome I, that exists at 8-10 copies per exponentially growing cell (Hansen, 1978). The approximate number of *merA* copies per cell in: R1 = 0; MD735 = 1; MD767 = 10; MD736 = 10; MD737 = 150. An exponentially growing *D. radiodurans* cell contains about five times the DNA content of an *E. coli* stationary-phase cell (Krasin et al., 1977). Taking this into consideration, it is estimated from data shown in Figure 9A, B that *E. coli* BL308 has about 20-30 *mer* copies per cell.

Resistance to mercury (II): The engineered *D. radiodurans* strains, as well as the *E. coli* strain BL308 (24), were inoculated into liquid medium containing 50 μM Merbromin or 50 μM $HgCl_2$ and growth for each was monitored over a period of thirteen days (Figure 9D). Wildtype *D. radiodurans* did not grow in the presence of 50 μM Hg (II) and was inhibited by 10-15 μM Merbromin or $HgCl_2$. The strains containing the cloned *mer* operon were variably affected by Hg (II) showing the following order of resistance: BL308 > MD737 > MD736 > MD735 > MD767 > R1 (Figure 9D). It should be noted that the normal growth rate of *E. coli* is about four times that of *D. radiodurans*. The five-day lag phase observed before the onset of growth of *mer*-containing *D. radiodurans* cells in 50 μM Hg (II) (Figure 9D) could be shortened to one day by inoculating Hg (II)-containing medium with cells pre-grown in the presence of 5 μM Hg (II). The growth rates and final cell densities of these pre-induced *D. radiodurans* cultures did not differ significantly from those shown in Figure 9D. This result supports that the *mer* genes are being induced in *D. radiodurans*. The most mercury resistant *D. radiodurans* strain MD737 was examined to determine the highest Hg (II) concentration at which growth

could be sustained. It was found that MD737 cells pre-cultured in Hg (II) could subsequently grow in medium containing up to 100 μM Hg (II) (data not shown).

Strain MD736 is more resistant to Hg (II) than MD767, even though they apparently have the same *mer* copy number (Figures 9A, 9B). The copy number of the tandem duplication- and direct- insertions in *D. radiodurans* are very similar (Daly *et al.*, 1995). As described above, direct insertions are much less versatile than tandem duplications and the strain MD736 is likely to be heterozygous; where some of the Chromosome I copies apparently lack the *mer* tandem duplication. The ability of strain MD736 to further increase its number of *mer* tandem duplications with mercury (II) selection was tested. By increasing the mercury concentration over a range of 0-35 μM, the copy number of the *mer* operon in MD736 doubled, compared to growth with just Km selection (Figure 9C). MD767, containing the direct *mer* insertion did not show a change in copy number with increasing mercury selection, compared to selection with Km (Figure 9C).

Effect of γ-radiation: To determine any effect of continuous exposure to γ-radiation on the growth of these strains in the presence of Hg (II), we tested the strains R1 (*recA*$^+$, *mer*$^-$), rec30 (*recA*$^-$, *mer*$^-$; Daly1), MD735 (*recA*$^+$, *mer*$^+$), MD736 (*recA*$^+$, *mer*$^+$), MD737 (*recA*$^+$, *mer*$^+$), MD767 (*recA*$^+$, *mer*$^+$), and the wildtype *E. coli* strain K12 (*recA*$^+$) containing pDB7 (BL308 [24]) (*mer*$^+$) (Figure 10). After four days of incubation in an irradiating (60 Gy/hr) and mercury (II)-containing environment, as expected, the only strains that could grow in the presence of both were MD735, MD736, MD737, and MD767 (Figure 10D).

Example 3

Construction of a toluene-metabolizing and Hg (II) resistant *D. radiodurans* strain MD764.

To assess the potential for expressing multiple remediating functions, encoded on separate gene cassettes, in *Deinococcus*, a strain expressing both mercury resistance/reducing and toluene metabolizing functions (Figure 9D, 11) was constructed. The genetic procedure of integrating two different gene cassettes at the same *D.*

radiodurans genomic locus has been described previously (Daly *et al.*, 1996), but in the context of analyzing recombination following high dose irradiation, and not gene expression.

Strain MD764 was analyzed for its ability to resist (Figure 9D) and reduce Hg (II) (Figures 12, 13) as well as metabolize the TDO specific substrate toluene (Figure 11D). The growth characteristics of strain MD764 in Hg (II), in the presence and absence of radiation (60 Gy/hr) were indistinguishable from those expressed in the parent strain MD737 (Figure 11C). MD764 could also reduce Hg (II) to Hg (0) (Figures 12, 13), in a manner similar to, if not indistinguishable from, MD737.

TDO activity was measured by ultraviolet (UV) absorbance and thin layer chromatography: Mercury (II), and the expression of *mer* operon genes, did not erode the ability of recombinant *D. radiodurans* cells to express functional toluene dioxygenase activity. This was demonstrated with strain MD764 when toluene was provided as the substrate (Figure 11D). Toluene dioxygenase oxidizes toluene to *cis*-1,2-dihydroxy-3-methylcyclohexa-3,5-diene (*cis*-toluene dihydrodiol), which absorbs maximally at 264 nm, and substantial absorbance at this wavelength was observed in culture supernatants of *D. radiodurans* MD764 (Figure 11D) containing the recombinant mercuric reductase and toluene dioxygenase genes (Figure 11B), but not in the wild-type strain *D. radiodurans* R1, lacking both of those gene cassettes (Figure 11D). From the extinction coefficient (33), the apparent dihydrodiol product was present at a concentration of about 1 mM at 31 hours. The putative product was greater at 31 hours than at 106 hours. These data were supported by direct observation of a product by TLC in comparison with authentic *cis*-toluene dihydrodiol (Figure 11D, inset). After 20 hours, a single metabolite (Figure 11D, inset, lane 2) comigrating with the dihydrodiol standard (Figure 11D, inset, lane 1) was observed. The amount of the dihydrodiol product decreased upon further incubation, consistent with the product data obtained by UV spectroscopy. The *cis*-dihydrodiol product of toluene dioxygenase is dehydrogenated by *D. radiodurans* to a product with a mass spectrum identical to 3-methylcatechol (unpublished). The data in Figure 10D (top) is consistent with this as the UV absorption maximum of the product(s) in the growth medium of strain MD764 shifts from that of a dihydrodiol (λ_{max} at 264 nm) at 31 hours to

that of a catechol (λ_{max} at 270 nm) at 106 hours.

Reduction of Hg (II) to volatile elemental Hg (0) by engineered strains.

Mercuric reductase assays: MerA activity was determined in cell extracts of *D. radiodurans* strains R1, MD767, MD735, MD736, MD737 and MD764 by following Hg (II)-stimulated NADPH oxidation spectrophotometrically (Schottel, 1978). Mercury (II)-dependent NADPH oxidation was observed in cell extracts of recombinant strains containing the *merA* gene, but not in wildtype *D. radiodurans* strain R1 (Figure 12A). In the absence of Hg (II), the rate of NADPH oxidation by the *mer* containing strains was comparable to that of strain R1 (Figure 12A, curve A). Also, there was good correlation between the variable Hg (II)-dependent NADPH oxidation activity (Figure 12A) and the resistance of strains to Hg (II) (Figure 9D).

MerA was visualized by SDS-polyacrylamide gel electrophoresis (PAGE) (Figure 12B). Cell extracts from strains R1, MD767, MD735, MD736, MD737, and MD764 were subjected to Orange A dye affinity chromatography as described previously (Schottel, 1978). After extensive column washing, the mercury-dependent NADPH oxidation activity was eluted from the column using NADPH; a process that increased the specific activity about 20-fold, and is an amount comparable to results from the same procedure used to purify MerA from other bacteria. SDS-PAGE analysis of the fractions containing MerA showed enrichment of two major proteins with approximate molecular weights of 62 kDa and 54 kDa in *merA*-containing strains, but not in the control strain R1 (wildtype). Two bands of similar molecular weights associated with mercuric reductase activity have been described for both the *E. coli* reductase (Nakamura *et al.*, 1988) and the *P. aeruginosa* enzyme (Schottel, 1978); the ratio of the two bands shifted depending on the storage of the protein, but with no significant change in specific activity. It is likely that the 54 kDa band is a proteolytic fragment of the larger.

Mercury volatilization: The observed mercuric ion dependent NADPH oxidation suggests the concomitant production of volatile Hg (0) by the *mer*-containing *D. radiodurans* strains. An X-ray film assay (38) (Figure 12C) was sued for detecting the

production of Hg (0) vapor by the incubation of the *D. radiodurans* strains with Hg (II). There was a good correlation of results between the Hg (II) resistance profiles of these engineered strains containing the *mer* operon (Figure 9D) and the X-ray film results (Figure 12C); elemental mercury vapor reacts with the silver ions of X-ray film, causing film darkening. Following 14 hours of strain incubation with Hg (II) in the microplate, covered with an X-ray film, only those strains containing the *mer* operon caused film exposure (Figure 12C). Incubation of cells with either Merbromin or $HgCl_2$ gave similar results, except that the film exposure was reduced when using Merbromin. Incubation of Hg (II) with the controls, R1 (wildtype), MD744 (*mer*$^-$, *tod*$^+$, Cm^r), MD560 (*mer*$^-$, *tod*$^+$, Km^r), or growth medium alone, did not show any evidence for Hg (0) volatilization.

Mercury depletion was determined in open cultures of *D. radiodurans* MD764 and the control strain R1 by cold vapor atomic fluorescence spectroscopy (CVAFS) (Figure 13). This experiment used CVAFS to detect total mercury in the system by analyzing the concentration of Hg (0) after quantitative reduction of Hg (II) to Hg (0), using stannous chloride. The bacterial cultures were open to the atmosphere for two hours before analysis, during which time biologically reduced Hg (II) would be anticipated to be lost from the system. The levels of mercury used were necessarily low because of the sensitivity of the CVAFS method. As shown in Figure 13, *D. radiodurans* strain MD764, representative of the *merA*-containing strains, was observed to expel mercury from the culture which it did to near baseline level in two hours. The control strain culture, lacking *merA*, showed no measurable loss of mercury over the same two hours. Although the cells were pre-incubated in medium without mercury for two hours and washed several times before the experiment was initiated, the level of mercury retained in the MD764 cells grown in the presence of $HgCl_2$ was more than twice the mercury added during the experiment. This sequestration of mercury did not significantly deplete over the course of the experiment. By increasing the length of the pre-incubation period to several hours and washing the cells exhaustively, the background level was reduced by up to 75%. However, the rate of mercury depletion was diminished to the level observed for cells not previously grown in the presence of Hg (II) (data not shown). Mercuric reductase specific activity in cell extracts from cells grown without Hg (II) is less than one half that of cell

extracts when the cells were grown in the presence of Hg (II) has also been observed.

Example 4

Development of *D. radiodurans* for Growth on/ Metabolism in DOE Mixed Wastes

Recombinant strains of *Deinococcus* engineered for bioremediation of mixed wastes may be prepared as a library of individual (primary) *Deinococcus* strains, each containing a different toxin-degrading gene cassette or metal resistance gene/s which has been cloned into Type-II, Type-III or Type-IV insertional vectors and separately transformed into *Deinococcus*. These primary stains are used in bioremediation compositions individually or combined depending on the composition of a particular waste site.

The primary isolates also serve as *Deinococcus* chromosomal reservoirs for toxin-remediating genes and metal resistance genes and provide a source of DNA that is subsequently transformed and combined into different strains to meet the requirements of surviving in and remediating a large variety of sites. For instance, genes necessary for metal resistance are cloned into *D. radiodurans* using Type-II (direct-insertion) vectors as described above. These metal resistance genes then become a permanent fixture in the cells' chromosomes and are not lost by recombinational 'pop-out' because the genes are be flanked by non-repetitive DNA. Because of the large variety of organic toxin-degrading genes available and the potential advantages of forming hybrid clusters that could be amplified for high level expression, integration of toxin-degrading genes into metal resistant strains is done using Type-III or Type-IV (duplication-insertion) vectors. Maintenance of the primary library strains is done with single antibiotic resistance markers and transformation derivatives containing different gene cassettes are maintained by multiple drug selection. In the case of cloning organic toxin-catabolizing genes into *D. radiodurans*, an alternative to selection with antibiotics is selection for a strain's ability to grow on or catabolize a specific organic substrate.

The ability of *D. radiodurans* to grow or catabolize one or more organic substrates, is selected for using minimal media (MM) supplemented with 0.0002% L-methionine in combination with required vitamins, minerals and nucleic acids as is known in the art. For

instance, MM is prepared as described above with the addition of toluene, biphenyl or metabolic intermediates of toxin degradation. This powerful selection, called 'shuffle-selection' allows *D. radiodurans* itself to 'choose' (by transformation and selection on MM plus a toxin) its own combination of genes that may allow growth. This gives the *D. radiodurans* strains the opportunity to construct its own metabolic pathways from the many catabolic genes presented with at the time of transformation. Further, by virtue of the repetitive chromosomal sequences flanking the duplication-insertion vectors, *D. radiodurans* amplifies those genes required for higher expression levels. The isolated strains that can grow on MM plus a toxin, may then be analyzed to determine what genes were selected and to what extent they were amplified for expression.

Example 5

D. geothermalis was transfected with plasmid pMD66 originally designed fro *D. radiodurans* (Daly, 1994a). pMD66 contains a *D. radiodurans* origin of replication and two distinct *Deinococcus* promoters from expressing cloned genes. These elements are functional in *D. geothermalis* growing at 50°C (Figure 14). DNA prepared from the indicated strains was digested with *Eco*RI, electrophoresed and subjected to Southern blotting with a radiolabeled pMD66 probe. Lanes: 1, λ phage cut with *Hind*III; 2, *D. geothermalis* wild type; 3, *D. radiodurans* R1; 4, *D. geothermalis + pMD66; 5,* D. radiodurans + pMD66; 6, purified pMD66 cut with *Eco*RI.

Although the present invention has been described in detail with reference to examples above, it is understood that various modifications can be made without departing from the spirit of the invention. Accordingly, the invention is limited only by the following claims. All cited patents and publications referred to in this application are herein incorporated by reference in their entirety.

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WHAT IS CLAIMED:

1. A radiation resistant bacterium engineered to detoxify at least one toxin.

2. A radiation resistant bacterium of claim 1 which survives acute exposure to ionizing radiation of up to about 15,000 Gy or can grow in the presence of continuous radiation of about 60 Gy/hour.

3. A radiation resistant bacterium of claim 1, wherein the bacterium is selected from the group consisting of *Enterococcus*, *Alcaligenes* and *Deinococcus*.

4. A radiation resistant bacterium of claim 1, wherein the toxin is selected from the group consisting of radionuclides, heavy metals and organic compounds.

5. A radiation resistant bacterium of claim 4, wherein the radionuclide is ^{235}Uranium $(\gamma, \alpha)^E$, ^{90}Strontium $(\beta^-)^E$, ^{238}Plutonium $(\alpha)^E$, ^{137}Cesium $(\gamma, \beta^-)^E$ and ^{99}Technetium $(\beta^-)^E$.

6. A radiation resistant bacterium of claim 4, wherein the heavy metal is chromium, lead, arsenic, zinc, cadmium, cobalt or mercury.

7. A radiation resistant bacterium of claim 4, wherein the organic compound is a chlorinated hydrocarbon, fuel hydrocarbon or polychlorinated biphenyl.

8. A radiation resistant bacterium of claim 7, wherein the chlorinated hydrocarbon is trichloroethylene.

9. A radiation resistant bacterium of claim 7, wherein the fuel hydrocarbon is toluene.

10. A radiation resistant bacterium of claim 7, wherein the polychlorinated biphenyl is Arochlor.

11. A radiation resistant bacterium of claim 1, wherein the toxin is selected from the group consisting of pesticides, toxicants, agricultural chemicals, algicides, fungicides, sterilants, polycyclic aromatic hydrocarbons, polychlorinated biphenyls, greasy wastes, solvents, crude oil, diesel fuel, waste oil, Bunker "C" oil, phenolics, halogenated hydrocarbons, terpene alcohols, anthracene, chlorotoluenes, chrysene, cresols, di-N-octylphthalate, dichlorobenzene, dichlorethanes, dichloropropanes, dichlorotoluene, 2-ethoxyethanol, ethylene glycol, ethylene glycol monoethyl ether acetate, ethylbenzene, fluorene, isoprenoids, methyl ethyl ketone, methylene chloride, naphthalene, pentachlorophenol, phenanthrene, 1,1,2,2-tetrachloroethane, toluene, 1,1,2-trichloroethane, trichloroethylene, benzoate, chlorobenzoates, methanol, ethyl acetate, cyclohexanone, ethylbenzene, 2,4-dichlorophenoxyacetic acid, 2,4,5-trichlorophenoxyacetic acid, m,o,p-xylene, butyl acetate, camphor, hexane, heptane, octane, nonane, d-limonene, linalool, geraniol, citronellol.

12. A radiation resistant bacterium of claim 1, wherein the bacterium has been engineered to express a heterologous protein or enzyme selected from the group consisting of toluene dioxygenase, the proteins encoded by the *mer* operon, the proteins encodes by the *Pseudmonas* Tol region, the proteins encoded by the *xyl*L-*xyl*E operon, a monooxygenase, the proteins encoded by *bphA1A2A3A4*, the proteins encoded by *czc*A, B and C genes, the *smt*A abd B genes and the *ars*A and B genes.

13. A radiation resistant bacterium engineered to detoxify at least two toxins.

14. A radiation resistant bacterium of claim 13 which survives acute exposure to ionizing radiation of up to about 15,000 Gy or can grow in the presence of continuous radiation of about 60 Gy/hour.

15. A radiation resistant bacterium of claim 13, wherein the bacterium is *Deinococcus radiodurans*.

16. A radiation resistant bacterium of claim 13, wherein the toxin is selected from the group consisting of radionuclides, heavy metals and organic compounds.

17. A radiation resistant bacterium of claim 16, wherein the radionuclide is ^{235}Uranium $(\gamma, \alpha)^E$, ^{90}Strontium $(\beta^-)^E$, ^{238}Plutonium $(\alpha)^E$, ^{137}Cesium $(\gamma, \beta^-)^E$ and ^{99}Technetium $(\beta^-)^E$.

18. A radiation resistant bacterium of claim 16, wherein the heavy metal is chromium, lead, arsenic, zinc, cadmium, cobalt or mercury.

19. A radiation resistant bacterium of claim 16, wherein the organic compound is a chlorinated hydrocarbon, fuel hydrocarbon or polychlorinated biphenyl.

20. A radiation resistant bacterium of claim 19, wherein the chlorinated hydrocarbon is trichloroethylene.

21. A radiation resistant bacterium of claim 19, wherein the fuel hydrocarbon is toluene.

22. A radiation resistant bacterium of claim 19, wherein the polychlorinated biphenyl is Arochlor.

23. A radiation resistant bacterium of claim 13, wherein the toxin is selected from the group consisting of pesticides, toxicants, agricultural chemicals, algicides, fungicides, sterilants, polycyclic aromatic hydrocarbons, polychlorinated biphenyls, greasy wastes, solvents, crude oil, diesel fuel, waste oil, Bunker "C" oil, phenolics, halogenated hydrocarbons, terpene alcohols, anthracene, chlorotoluenes, chrysene, cresols, di-N-octylphthalate, dichlorobenzene, dichlorethanes, dichloropropanes, dichlorotoluene, 2-ethoxyethanol, ethylene glycol, ethylene glycol monoethyl ether acetate, ethylbenzene, fluorene, isoprenoids, methyl ethyl ketone, methylene chloride, naphthalene, pentachlorophenol, phenanthrene, 1,1,2,2-tetrachloroethane, toluene, 1,1,2-trichloroethane, trichloroethylene, benzoate, chlorobenzoates, methanol, ethyl acetate, cyclohexanone, ethylbenzene, 2,4-dichlorophenoxyacetic acid, 2,4,5-trichlorophenoxyacetic acid, m,o,p-xylene, butyl acetate, camphor, hexane, heptane, octane, nonane, d-limonene, linalool, geraniol, citronellol.

24. A radiation resistant bacterium of claim 13, where the bacterium has been engineered to express a heterologous protein or enzyme selected from the group consisting of toluene dioxygenase, the proteins encoded by the *mer* operon, the proteins encodes by the *Pseudmonas* Tol region, the proteins encoded by the *xyl*L-*xyl*E operon, a monooxygenase, the proteins encoded by *bphA1A2A3A4*, the proteins encoded by *czc*A, B and C genes, the *smt*A abd B genes and the *ars*A and B genes.

25. A bioremediation composition comprising a bacterium of any one of claims 1-24.

26. A bioremediation composition of claim 25 further containing an agent selected from the group consisting of a film forming agent and a nutrient agent.

27. A bioremediation composition of claim 25 which if formulated for controlled release.

28. A bioremediation composition of claim 26 which if formulated for controlled release.

29. A *Deinoccocus radiodurans* strain which metabolizes toluene and is resistant to mercury.

30. The *Deinoccocus radiodurans* strain of claim 29 which expresses the *P. putida todC1C2BA* and *E. coli merA* operons.

31. A method of bioremediation, comprising the step of exposing a sample to a composition of 25.

32. A method of bioremediation, comprising the step of exposing a sample to a composition of any one of claims 26-28.

33. A method of claim 32, wherein the sample is contaminated with radionuclides.

34. A method of claim 33, wherein the composition is released into a waste site.

35. A bioremediation composition comprising a bacterium of claim 30.

36. A bioremediation composition of claim 35 further containing an agent selected from the group consisting of a film forming agent and a nutrient agent.

37. A bioremediation composition of claim 35 which if formulated for controlled release.

38. A method of bioremediation, comprising the step of exposing a sample to a

composition of any one of claims 35-37.

39. A method of claim 38, wherein the sample is contaminated with radionuclides.

40. A method of claim 39, wherein the composition is released into a waste site.

41. A radiation resistant bacterium of any one of claims 1-4 and 16-24, wherein the bacterium is selected from the group consisting of *D. radiodurans*, *D. radiopugnans*, *D. grandis*, *D. proteolyticus*, *D. murrayi*, *D. geothermalis*, and *D. radiophilus*.

ABSTRACT

The invention relates to radiation resistant bacteria engineered to detoxify at least one toxin and preferably several toxins. Radiation resistant bacteria of the invention include *Deinococcus* strains engineered to detoxify toxins such as radionuclides, heavy metals and organic compounds. The invention also includes bioremediation compositions comprising at least one radiation resistant bacterial strain capable of detoxifying toxins and methods relating to the preparation and use of such compositions. Such compositions and methods may be utilized to detoxify industrial waste sites contaminated with radioactivity.

COMBINED DECLARATION FOR PATENT APPLICATION AND POWER OF ATTORNEY

U.S. Department of Commerce
Patent and Trademark Office
Attorney Docket **044508-5003**

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name,

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled:

ENGINEERED RADIATION RESISTANT BIOREMEDIATING BACTERIA

was filed as PCT International Application No. **PCT/US00/26504** on **September 27, 2000**.

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose to the U.S. Patent and Trademark Office information which is material to the patentability of claims presented in this application in accordance with Title 37, Code of Federal Regulations, 1.56.

I hereby authorize and request the attorneys empowered in this Declaration and Power of Attorney to insert above the serial number of the application identified above when known.

I hereby claim foreign priority benefits under Title 35, United States Code, 119(a)-(d) or 365(b) of any foreign application(s) for patent or inventor's certificate or 365(a) of any PCT international application(s) designating at least one country other than the United States of America listed below and have also identified below any foreign application(s) for patent or inventor's certificate or any PCT international application(s) designating at least one country other than the United States of America filed by me on the same subject matter having a filing date before that of the application(s) of which priority is claimed:

Prior Foreign Applications

Country	Application Number	Filing Date	Priority Claimed
			☐ Yes ☐ No

I hereby claim the benefits under Title 35, United States Code 119(e) of any U.S. provisional applications listed below.

U.S. Provisional Applications

U.S. Provisional Application No.	U.S. Filing Date
60/155,767	**September 27, 1999**

I hereby claim the benefit under Title 35, United States Code, 120 of any United States application(s) or 365(c) of any PCT international application(s) designating the United States of America that is/are listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in that/those prior application(s) in the manner provided by the first paragraph of Title 35, United States Code, 112, I acknowledge the duty to disclose to the U.S. Patent and Trademark Office all information known to me to be material to the patentability of claims presented in this application in accordance with Title 37, Code of Federal Regulations, 1.56 which became available between the filing date of the prior application(s) and the national or PCT international filing date of this application:

Combined Declaration For Patent Application and Power of Attorney - **(Continued)** Attorney Docket **044508-5003**
(includes Reference to PCT International Applications)

Prior U.S. Applications or PCT Applications Designating the U.S. for Benefit:

U.S. Applications		Status		
U.S. Application No.	U.S. Filing Date	Patented	Pending	Abandoned

PCT Applications Designating the U.S.		Status		
PCT Application No.	International Filing Date	Published	Pending	Abandoned

POWER OF ATTORNEY: As a named inventor, I hereby appoint the registered practitioners of Morgan, Lewis & Bockius LLP included in the Customer Number provided below to prosecute this application and to transact all business in the Patent and Trademark Office connected therewith, and direct that all correspondence be addressed to that Customer Number.

Customer Number: 009629

Direct Telephone Calls To:
(name and telephone number)

Michael Tuscan, Ph.D.
202-739-5870

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Full Name of First Inventor	**Michael J. Daly**	
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Second Inventor's Signature	Lawrence P. Wackett	DATE July 29, 2002

Listing of Inventors Continued on attached pages ☐Yes ☒No

1-WA/1730631.1

COMBINED DECLARATION FOR PATENT APPLICATION AND POWER OF ATTORNEY

U.S. Department of Commerce
Patent and Trademark Office
Attorney Docket **044508-5003**

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name,

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled:

ENGINEERED RADIATION RESISTANT BIOREMEDIATING BACTERIA

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Prior Foreign Applications

Country	Application Number	Filing Date	Priority Claimed
			☐ Yes ☐ No

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U.S. Provisional Applications

U.S. Provisional Application No.	U.S. Filing Date
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Combined Declaration For Patent Application and Power of Attorney - **(Continued)**
(includes Reference to PCT International Applications)

Attorney Docket **044508-5003**

Prior U.S. Applications or PCT Applications Designating the U.S. for Benefit:				
U.S. Applications		Status		
U.S. Application No.	U.S. Filing Date	Patented	Pending	Abandoned
PCT Applications Designating the U.S.		Status		
PCT Application No.	International Filing Date	Published	Pending	Abandoned

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I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

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